In Vitro Exposure Systems and Dosimetry Assessment Tools for Inhaled Tobacco Products: Workshop Proceedings, Conclusions and Paths Forward for *In Vitro* Model Use

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Summary — In 2009, the passing of the *Family Smoking Prevention and Tobacco Control Act* facilitated the establishment of the FDA Center for Tobacco Products (CTP), and gave it regulatory authority over the marketing, manufacture and distribution of tobacco products, including those termed 'modified risk'. On 4–6 April 2016, the Institute for In Vitro Sciences, Inc. (IIVS) convened a workshop conference entitled, *In Vitro Exposure Systems and Dosimetry Assessment Tools for Inhaled Tobacco Products*, to bring together stakeholders representing regulatory agencies, academia and industry to address the research priorities articulated by the FDA CTP. Specific topics were covered to assess the status of current *in vitro* smoke and aerosol/vapour exposure systems, as well as the various approaches and challenges to quantifying the complex exposures in *in vitro* pulmonary models developed for evaluating adverse pulmonary events resulting from tobacco product exposures. The four core topics covered were: a) Tobacco Smoke and E-Cigarette Aerosols; b) Air–Liquid Interface-*In Vitro* Exposure Systems; c) Dosimetry Approaches for Particles and Vapours/*In Vitro* Dosimetry Determinations; and d) Exposure Microenvironment/Physiology of Cells. The 2.5-day workshop included presentations from 20 expert speakers, poster sessions, networking discussions, and breakout sessions which identified key findings and provided recommendations to advance these technologies. Here, we will report on the proceedings, recommendations, and outcome of the April 2016 technical workshop, including paths forward for developing and validating non-animal test methods for tobacco product smoke and next generation tobacco product aerosol/vapour exposures. With the recent FDA publication of the final deeming rule for the governance of tobacco products, there is an unprecedented necessity to evaluate a very large number of tobacco-based products and ingredients. The questionable relevance, high cost, and ethical considerations for the use of *in vivo* testing methods highlight the necessity of robust *in vitro* approaches to elucidate tobacco-based exposures and how they may lead to pulmonary diseases that contribute to lung exposure-induced mortality worldwide.

Key words: *computational fluid dynamics, COPD, dosimetry, e-cigarette exposure systems, ex vivo lung models, in vitro, in vitro lung, in vitro models, non-animal alternatives, pulmonary models, reconstructed epithelium airway models, tobacco exposure systems, tobacco regulatory science.*

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Introduction

The *Family Smoking Prevention and Tobacco Control Act* of 2009 established the Food and Drug Administration Center for Tobacco Products (FDA CTP)[b] and gave it regulatory authority over the marketing, manufacture and distribution of tobacco products in the United States. Included are those described as Modified Risk Tobacco Products (MRTPs). With the publication of the final deeming rule on 5 May 2016, the CTP has expanded the scope of regulatory jurisdiction to cover a wider range of tobacco products including pipe tobacco, cigars, electronic nicotine delivery systems, liquid nicotine, and hookah tobacco, affecting both large and small manufacturers and vendors of these tobacco products. In addition to defining the premarket submission requirements, it addresses ingredients (e.g. harmful and potentially harmful ingredients) found within tobacco products and the requirement to submit scientific evidence concerning the potential toxicity of a MRTP. Information related to the potential toxicities is addressed in a 2011 report, *Scientific Standards for Studies on Modified Risk Tobacco Products*, generated with input from the Institute of Medicine, which advised the FDA to require companies wishing to market an MRTP to include information on the "human health risks of the MRTP, including the risk of tobacco-related diseases…"

Much of the information on health risks is traditionally interpreted from toxicological experiments conducted on animals. However, the human relevance of the respiratory toxicology data obtained from animals exposed to tobacco smoke has been called into question, as specific human respiratory tract lesions are rarely exhibited in the rodent models (1–3), with some exceptions in rodent models particularly sensitive to tobacco smoke inhalation (4–6). However, concerns for *in vivo* research remain due to experimental costs, as well as differences in the physiology and breathing behaviours between humans and rodents, the deposition and exposure of inhaled particulates, droplets, and vapours throughout the rodent respiratory tract that may poorly model actual human exposures.

The 2007 report, *Toxicity Testing in the 21st Century — A Vision and a Strategy* (7), describes a path forward for toxicology in general and envisions the use of more human-relevant and predictive *in vitro* models for estimating human health risks. With the increased use of *in vitro* pulmonary tissue models comes the concomitant need for different exposure and dosimetry methods than are traditionally used in animal inhalation studies. While use of these new *in vitro* approaches is becoming common within the tobacco industry and research institutions, their relevance and utility has not yet been well established or publicised within many sectors of the regulatory community. This will certainly inhibit the use and acceptance of *in vitro* approaches in regulatory submissions for new MRTPs. In an effort to highlight the potential usefulness of such methods in assessing human health risk within a regulatory framework and to help harmonise, within industry and academic research laboratories, exposure and dosimetry approaches for *in vitro* systems, the Institute for In Vitro Sciences, Inc. (IIVS) convened a workshop covering these topics in April 2016. The workshop theme and subject areas to be explored were developed with input from numerous stakeholders, many of which attended IIVS' first in a series workshop held in December 2014, *Assessment of In Vitro COPD [Chronic Obstructive Pulmonary Disease] Models for Tobacco Regulatory Science*, the proceedings of which are published (8).

Guidance received was from experts in tobacco research (government, academia, tobacco industry, and independent groups), instrument and product manufacturers, and *in vitro*/*ex vivo* model scientists knowledgeable of the published FDA-CTP research priorities, and the current challenges in making accurate assessments of inhaled tobacco product-induced pulmonary risk.

The IIVS workshop series was conceived and developed based on identified needs of the FDA-CTP, as evidenced by the public dissemination of their research priorities (https://grants.nih.gov/grants/guide/notice-files/NOT-CA-12-007.html) and by researchers from different sectors who are interested in better understanding the adverse health effects of tobacco products. The workshop series is meant to address at least portions of the following specific FDA-CTP research priorities:

— What *in vitro* and *in vivo* assays are capable of comparative toxicity between two different tobacco products; with special attention to cardiotoxicity, respiratory toxicity, carcinogenicity, and developmental/reproductive toxicity?

— What other constituents, compounds, design features, and tobacco use behaviours impact the toxicity and carcinogenicity of tobacco products and smoke?

The first workshop (8–10 December 2014) focused on *in vitro* systems and how they can contribute to a better understanding of key pulmonary events that may lead to COPD. It was during the interactive discussions and breakout sessions where it became evident that employing specific metrics to establish dose–response relationships for *in vitro*/*ex vivo* human models of the lung would be a challenge using current exposure systems. Further interactions between IIVS organisers and stakeholders allowed the refinement of the next-in-series workshop topics that would address this issue.

[b]*The abbreviations used throughout the report are also listed in Table A1 of the Appendix.*

These discussions resulted in a third workshop, *In Vitro Exposure Systems and Dosimetry Assessment Tools for Inhaled Tobacco Products*, which was held on 4–6 April 2016, in Bethesda, Maryland (USA). It was attended by 74 stakeholders, including regulators, industry, biotechnology providers, research institutions and the animal protection community. The 2.5-day programme consisted of four core subject areas covered during presentations by 20 experts in the field (see Table 1), and 15 posters which addressed a wide scope of topics relevant to tobacco-based exposure and dosimetry using *in vitro* systems. The four core areas were: a) Tobacco Smoke and E-Cigarette Aerosols; b) Air–Liquid Interface-*In Vitro* Exposure Systems; c) Dosimetry Approaches for Particles and Vapours/*In Vitro* Dosimetry Determinations; and d) Exposure Microenvironment/ Physiology of Cells. Breakout group sessions were held for two of the four core topics and were intended for consolidating current views on *in vitro* exposure systems and the dosimetry approaches that should be considered for standardisation, and identifying areas that require additional research and/or development.

Topics from the workshop and the conclusions from the breakout groups are presented in the following sections.

Introductory Presentations

Welcome and Overview *(Holger Behrsing, IIVS; Erin Hill, IIVS; and Raymond Tice, NIEHS [retired])*

This programme, organised by the Institute for In Vitro Sciences (IIVS), explored *in vitro* exposure systems and dosimetry assessment tools for inhaled tobacco products. Its purpose was to highlight the current status of *in vitro* to *in vivo* correlations, whole tobacco smoke and e-cigarette aerosol/vapour constituents, *in vitro* exposure systems, dosimetry approaches, the exposure microenvironment, and promising technologies that may advance science in these areas.

To date, much of the research and testing in respiratory and inhalation toxicology has focused on the use of animal models. With the development of new technologies, such as reconstructed human airway tissues, researchers are turning their attention to *in vitro* assessments.

In this workshop, invited experts from industry, government, academia and non-profits presented talks and posters covering key areas in exposure and dosimetry for non-animal testing. Its intent was to facilitate an exchange of information for a better understanding of exposure systems, and to discuss the methodology that best captures what is delivered to the *in vitro* systems used to assess human-relevant biological responses.

This 2.5-day workshop was the third in a series of respiratory toxicology workshops organised by IIVS. The first was held in December 2014, followed by a second technical workshop in June 2015 that addressed conclusions drawn from the first laboratory exercises; the proceedings and conclusions of the first workshop were published in 2016 (8).

Advancing Regulatory Science at the US FDA with More Predictive Models *(Suzanne Fitzpatrick, US Food and Drug Administration)*

Suzanne Fitzpatrick reviewed the efforts of the US Food and Drug Administration (FDA) to advance regulatory science and toxicology.

Toxicology is an area of science important to the FDA's ability to predict product safety or assess the potential significance of chemicals in products or the environment. Advances in toxicology testing — such as systems biology, stem cells, engineered tissues, computerised modelling — create unique opportunities to transform this predictive science to bring needed products to people faster and more safely, and to replace, reduce and/or refine animal testing.

The FDA is working toward transitioning new 21st century technologies to enhance the efficiency and effectiveness of chemical risk management. Currently, the FDA relies heavily on animal studies, and generates information for all possible outcomes, based on traditional toxicity tests. Future goals include less reliance on animal studies and more-tailored data generation, based on an understanding of toxicity pathways.

In 2008, a Memorandum of Understanding (MoU) was issued on *High-throughput Screening, Toxicity Pathway Profiling, and Biological Interpretation of Findings*, and has brought together four federal agencies: the Environmental Protection Agency (EPA), the National Center for Advancing Translational Sciences (NCATS), the National Institute of Environmental Health Sciences (NIEHS), and the FDA. Known informally as Tox21 (Toxicology in the 21st Century), the MoU was renewed last summer and now covers all types of *in vitro* testing, including organs-on-a-chip. Its goals are to identify patterns of compound-induced biological response, in order to characterise toxicity/disease pathways, facilitate cross-species extrapolation and model low-dose extrapolation. Tox21 also aims to prioritise compounds for more extensive toxicological evaluation and develop predictive models for biological response in humans.

In the area of regulatory safety assessment, the FDA recognises the need for new approaches that are more predictable, more reliable, faster and less expensive. In 2010, the FDA and the National Institutes of Health (NIH) collaborated to launch

Table 1: Summary of contents

the *Advancing Regulatory Science Initiative*, an effort designed to accelerate the process from scientific breakthrough to the availability of new, innovative medical therapies for patients. Specifically, the FDA is looking for better models of assessing human adverse response. Although 'adverse' means different things to each FDA centre, and each has a different way of approaching problems, all are interested in a better understanding of toxicity mechanisms at multiple levels of biological organisation — including genes, proteins, pathways and cell/organ function. The FDA is also exploring methods to characterise molecular targets and host genetic factors that might be associated with rare and unexpected adverse events.

One of the main challenges to progress in this area is the regulators' reluctance to rely on new technology methods for product approvals. There is a long history of the generally successful use of traditional animal testing methods, and a lack of confidence in non-animal methods. To address this roadblock, the FDA has been sponsoring workshops to help regulators learn about new technologies.

Other important FDA collaborations include a partnership with the NIH and the Defense Advanced Research Projects Agency (DARPA), to jointly develop new tools that can be used in therapeutic development. The *FDA-DARPA-NIH Microphysiological Systems Program* was started in 2011 to support the development of human microsystems, or organ-chips, to screen for safe and effective drugs swiftly and efficiently before human testing. The human-on-a-chip will be developed with at least ten organs, all linked together and viable for four weeks.

The FDA is also awaiting the results of the *National Research Council's Second Meeting of the Committee on Incorporating 21st Century Science into Risk-based Evaluations*. This report will combine the recommendations of two reports, *Toxicity Testing in the 21st Century* and *Exposure Testing in the 21st Century*, and will focus on incorporating 21st century science-based risk strategies into risk assessment.

Another major challenge with the paradigm shift to new methodologies is to establish scientific validation — the process concerned with assessing assumptions, relevance, reliability, reproducibility, and sensitivity of tests for particular purposes, and understanding uncertainties. Because 'validation' means different things to different centres, a 'one size fits all' approach is not an option. Key points are:

— The FDA is working toward transitioning new 21st century technologies, to enhance the efficiency and effectiveness of chemical risk management.

— Currently, the FDA relies heavily on animal studies, and generates information for all possible outcomes, based on traditional toxicity tests. Future goals include less reliance on animal studies and tailored data generation, based on understanding of toxicity pathways.

— Investments in toxicology and regulatory science can enable the FDA to better protect and promote the health of people in the USA and throughout the world.

— Collaboration is essential to define needed pathways and catalyse change.

In Vitro to *In Vivo* Extrapolation in Humans *(Robert Devlin, US Environmental Protection Agency)*

Robert Devlin discussed the need for using *in vitro* methods for toxicity testing, the actions of the Environmental Protection Agency (EPA) and other agencies to address this need, and the challenges of assessing how well *in vitro* assays might predict human *in vivo* responses.

Recognising that traditional animal toxicity studies are only feasible for a small percentage of the thousands of chemicals that must be assessed for risk, the EPA asked the National Research Council (NRC) to develop a long range vision and a strategy to advance toxicity testing. The NRC's 2007 report concluded that the only way to screen the increasing number of environmental chemicals regulated is by a conversion to *in vitro* techniques. The NRC recommended the expanded use of *in vitro* toxicity pathway data based on mode-of-action or adverse outcome pathway information.

The EPA established the National Center for Computational Toxicology (NCCT) to develop new software and methods for predictive toxicology. Among the research efforts managed by the NCCT is ToxCast™, a multi-year effort that uses high-throughput assays to screen cells for changes in biological activity that may suggest potential toxic effects. Toxicology in the 21st Century (Tox21), a collaboration of several federal agencies, is currently screening about 10,000 chemicals using a high-throughput robotic system that can assay about 150,000 wells in one day. However, inhaled toxicants are largely missing from the Tox21 assays due to the robots' inability to deal with compounds in a vapour or gaseous form.

Before *in vitro* toxicity pathway information can be useful in risk assessment, many challenges must be overcome. A quantitative relationship must be established between perturbation of a pathway following *in vitro* exposure and the downstream endpoints (i.e. pathophysiological changes at the tissue or organism level following *in vivo* exposure of animals or humans).

For *in vitro* testing of inhaled toxicants, models based on human primary lung cells are considered ideal for several reasons. These cells are the first

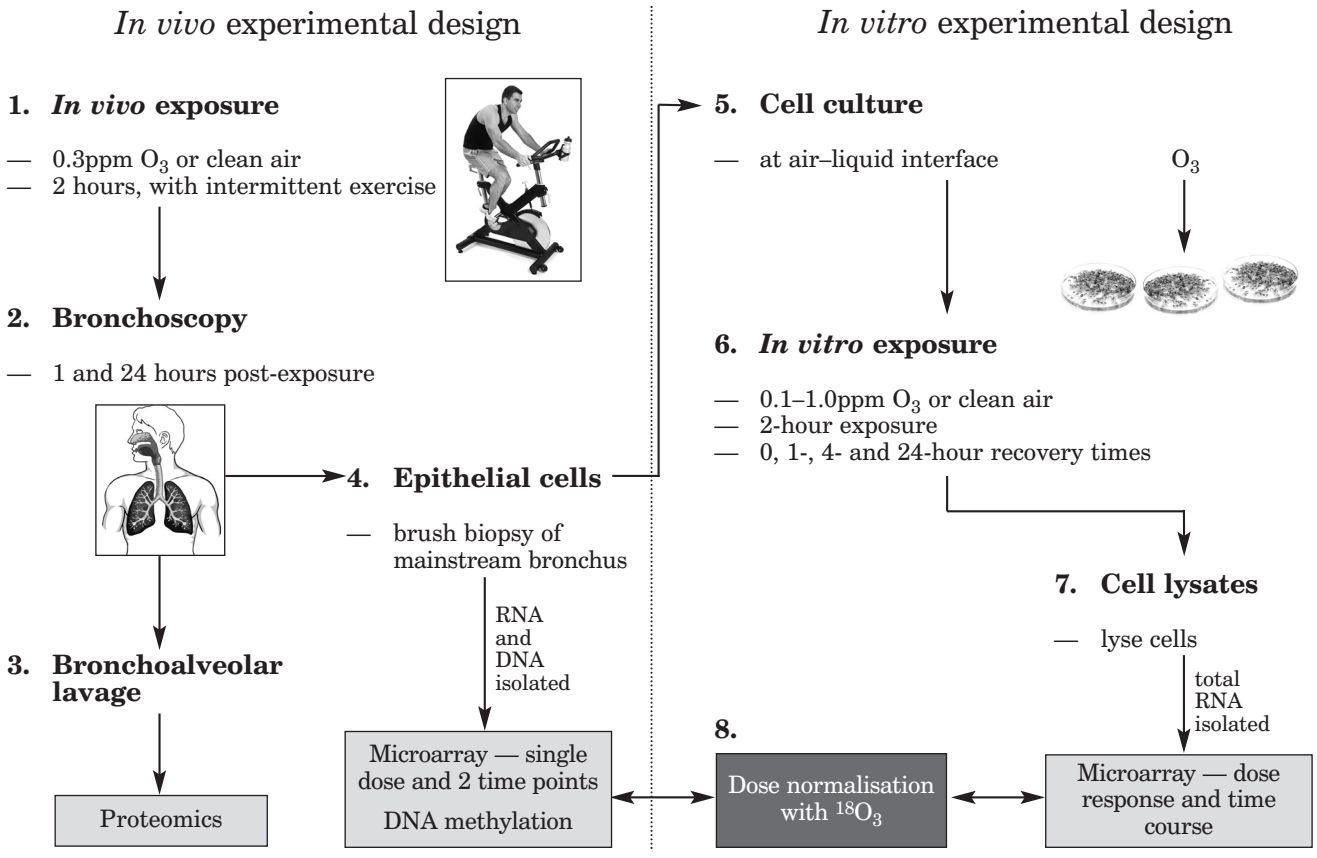
targets of inhaled toxicants and animal-to-human extrapolation is not needed. Primary cells respond more 'realistically' than immortalised or transformed cells, for example, in the induction of an inflammatory response. Primary cells also offer the possibility of examining genetic/epigenetic, disease, age and other factors, as well as intra-individual variability. In the near future, human airway epithelial and alveolar cells, derived from human pluripotent stem cells (iPSCs), are expected to become available for research. These cells retain all the advantages of primary cells, but have unlimited self-renewal.

In an effort to determine how well *in vitro* exposure models predict *in vivo* responses, the EPA launched the *Next Generation (NexGen) of Risk Assessment* effort, a multi-year collaboration among several organisations (9). NexGen selected ozone (O_3) as one of the prototype compounds for validation studies, because of the extensive human toxicity data available. More than 100 studies have shown that exposure to O_3 results in decrements in lung function, increases in markers of pulmonary inflammation, and alterations in host defence against inhaled pathogens.

Researchers in the NexGen ozone project identified and compared ozone-induced toxicity pathways following both *in vivo* and human *in vitro* exposures, to validate how well *in vitro* toxicity pathway information can predict human *in vivo* responses. Ozone labelled with the heavy oxygen isotope (^{18}O) was used for both exposures, to ensure that the dose of ozone attacking the cells *in vitro* was the exact same as the dose attacking them *in vivo* (10). The experimental design is summarised in Figure 1. Volunteers were exposed to 0.3ppm ozone or clean air on two separate occasions. At 1 hour and 24 hours after exposure to ozone, epithelial cells were recovered by brush scraping during bronchoscopy. RNA and DNA were isolated and toxicity pathways identified by microarray. Cells obtained following exposure to clean air were cultured and exposed to varying doses of ozone *in vitro* by using air–liquid interface exposure. RNA was collected at various times after exposure and toxicity pathways identified by microarray.

The results showed that *in vivo* more genes were differentially expressed at 1 hour compared with 24 hours post-exposure. There was very little overlap in the genes induced by ozone at 1 hour and 24 hours post-exposure. The microarray data for the *in vitro* results showed that, as the dose increased, the number of mRNAs whose concentration was

Figure 1: A summary of the experimental design



affected increased. Inflammation was activated at all three concentrations. A stress response and apoptosis were observed at the higher concentrations, but not at the lower ones. So, as the dose increased, the cells responded differently. When comparing the 11 networks activated *in vitro* with the 14 networks activated *in vivo* at the 1 hour timepoint, a number of networks overlapped, including those involved in the inflammatory response, immunological diseases and conditions, cellular movement, cellular growth and proliferation, cellular function and maintenance, immune cell trafficking, infectious diseases, and cell-to-cell signalling and interaction (unpublished data).

In conclusion, the preliminary data from the ozone project showed that the inflammation following *in vitro* exposure to ozone might be predictive of the inflammation seen following human exposure to ozone. From a qualitative point of view, the *in vitro* responses to ozone appeared to represent pretty well what happens downstream *in vivo*. The qualitative data generated from this study will feed into quantitative physiologically-based pharmacokinetic (PBPK) models to demonstrate the predictive value of *in vitro* toxicity testing. Expansion of this approach beyond ozone to other toxicants is needed. To summarise, the key points are:

— The challenges of evaluating thousands of chemicals while considering the cumulative effects of mixtures and limiting the use of animal testing has led to new approaches to toxicity testing.

— Conversion to *in vitro* techniques is the only way to screen the ever increasing number of environmental chemicals that must be regulated.

— Technologies that can transform existing approaches include high-throughput techniques, systems biology approaches and bioinformatics.

— Preliminary data from the NexGen ozone study showed that the inflammation seen following *in vitro* exposure to ozone might be predictive of inflammation seen following human exposure to ozone.

In Vitro Models for Tobacco Regulatory Science: Collaborative Efforts in Respiratory Toxicology *(Hans Raabe, IIVS)*

Hans Raabe summarised the outcomes of two recent collaborative efforts that explored the use of *in vitro* assays for making regulatory decisions for tobacco products.

Recognising that collaborative engagement would be key to the successful development and validation of these test methods, the Institute for In Vitro Sciences (IIVS) seeks to bring together experts from industry, regulatory agencies, academia and other stakeholders to identify, optimise and validate *in vitro* test methods for eventual tobacco product regulatory submissions. The first two workshops were held in December 2014 (Bethesda, MD) and June 2015 (Gaithersburg, MD).

The first 'informational' workshop was designed to marshal the current expertise to present upon the aetiology of chemical-induced chronic obstructive pulmonary disease (COPD), and the putative *in vitro* cell-based methods associated with key biological events leading to COPD. This workshop was conceptualised as an approach to evaluate two of the components of the FDA's Center for Tobacco Products (CTP) priorities set in 2012:

— What *in vitro* and *in vivo* assays are capable of comparative toxicity between two different tobacco products; with special attention to cardiotoxicity, respiratory toxicity, carcinogenicity, and developmental/reproductive toxicity?

— What constituents, compounds, design features, and tobacco use behaviours impact toxicity and carcinogenicity of tobacco products and smoke?

Workshop participants were asked to examine the current status of *in vitro/ex vivo* models and the ability of the models to predict toxicological outcomes relevant to COPD, and to propose research strategies. Key biological events that were identified within the adverse outcome pathway (AOP) of COPD included: inflammation and oxidative stress, ciliary dysfunction and ion transport, goblet cell hyperplasia and mucus production, and parenchymal/bronchial tissue destruction and remodelling.

In breakout sessions, participants regarded deficient mucociliary clearance as one of the key tissue-level events relevant to the clinical manifestation of tobacco-induced COPD. Similarly, changes in goblet cell morphology and mucus production were also considered to be key tissue-level events in the pathogenesis of COPD, and all were considered to be notably downstream in the AOP to more likely be predictive of COPD. Based upon these revelations, the breakout group recommended the subsequent optimisation and standardisation of these prototypic tools, in order to validate them for regulatory toxicology. Participants also acknowledged that, in order to adequately evaluate the reproducibility of the biological responses of the *in vitro* test systems to toxicants, reference chemicals should be selected and applied directly onto the tissue systems by using standard dose application methods, rather than non-standardised whole smoke/vapour exposure methods.

The subsequent technical workshop brought together the technical experts versed in the key areas identified in the first informational work-

shop's breakout sessions, including ciliary beating frequency, changes in goblet cell hyperplasia and mucus production. Workshop participants discussed current methodologies and whether basic protocols or test methods could be designed in a standardised approach that could be optimised and transferred to multiple laboratories. Activities focused on developing the test method protocols to evaluate the proof-of-principle concept. Work is currently underway at multiple laboratories to conduct the proof-of-principle protocols. The next steps will be to share and review the data, to evaluate the proof-of-principle hypotheses, and subsequently to optimise the protocols.

In summary, the first workshops identified various areas for cooperative development and optimisation of *in vitro* test methods, tissue models and endpoints relative to *in vivo* observations. The current informational workshop was designed to present on the state-of-the-art of *in vitro* respiratory tissue exposure and dosimetry technologies and techniques, to identify applications, comparisons of *in vivo* and *in vitro* dosimetry measurements, limitations and knowledge gaps, and propose activities to characterise and standardise these methodologies. Future activities will be targeted at reuniting standardised whole smoke/vapour exposure and dosimetry systems with optimised *in vitro* tissue models and endpoints for regulatory tobacco toxicology. The key points are:

— Developing and validating new test methods for use in a regulatory safety testing arena requires the input and guidance from multiple stakeholders.

— Identifying the regulatory requirements to address with testing may best be achieved through collaborative goal-oriented discussions between industry and regulatory community representatives, while the relevant scientific methods may be proposed and discussed by industrial and academic experts versed in the technologies.

Introduction to Exposure and Dosimetry

Species Differences in Respiratory Anatomy *(Kent Pinkerton, Center for Health and the Environment, University of California, Davis)*

Kent Pinkerton described the differences, as well as the similarities, that occur in the respiratory anatomy of mammalian species.

Although the size of the respiratory system varies widely across the mammalian species, the airway trees of most mammals share fairly similar branching patterns. All species show symmetric branching — with uniform divisions going from a parent airway to two daughter airways — as well as asymmetric branching, characterised by major and minor daughter airways (11, 12).

Typically, every mammalian species has most of the more than 40 different cell types found in the respiratory system, with many of them found within the airway epithelium. However, the cell populations, density, composition, distribution and metabolic potential, as well as the number and extent of the epithelial derivatives, vary according to the species.

Going deeper down into the lung, more cilia are seen along the conducting airway. Club cells, seen as dome-shaped cells by electron microscopy, occur in all mammalian species, and are involved in metabolism and immune function. Studies are underway to examine species-specific differences in club cells. Mucus cells, whether isolated from primary tissues or in an immortalised cell line, are an important cell type to keep in mind when going from *in vivo* to *in vitro* methodologies. Mucus velocity decreases with age across species due to reduced ciliary motility.

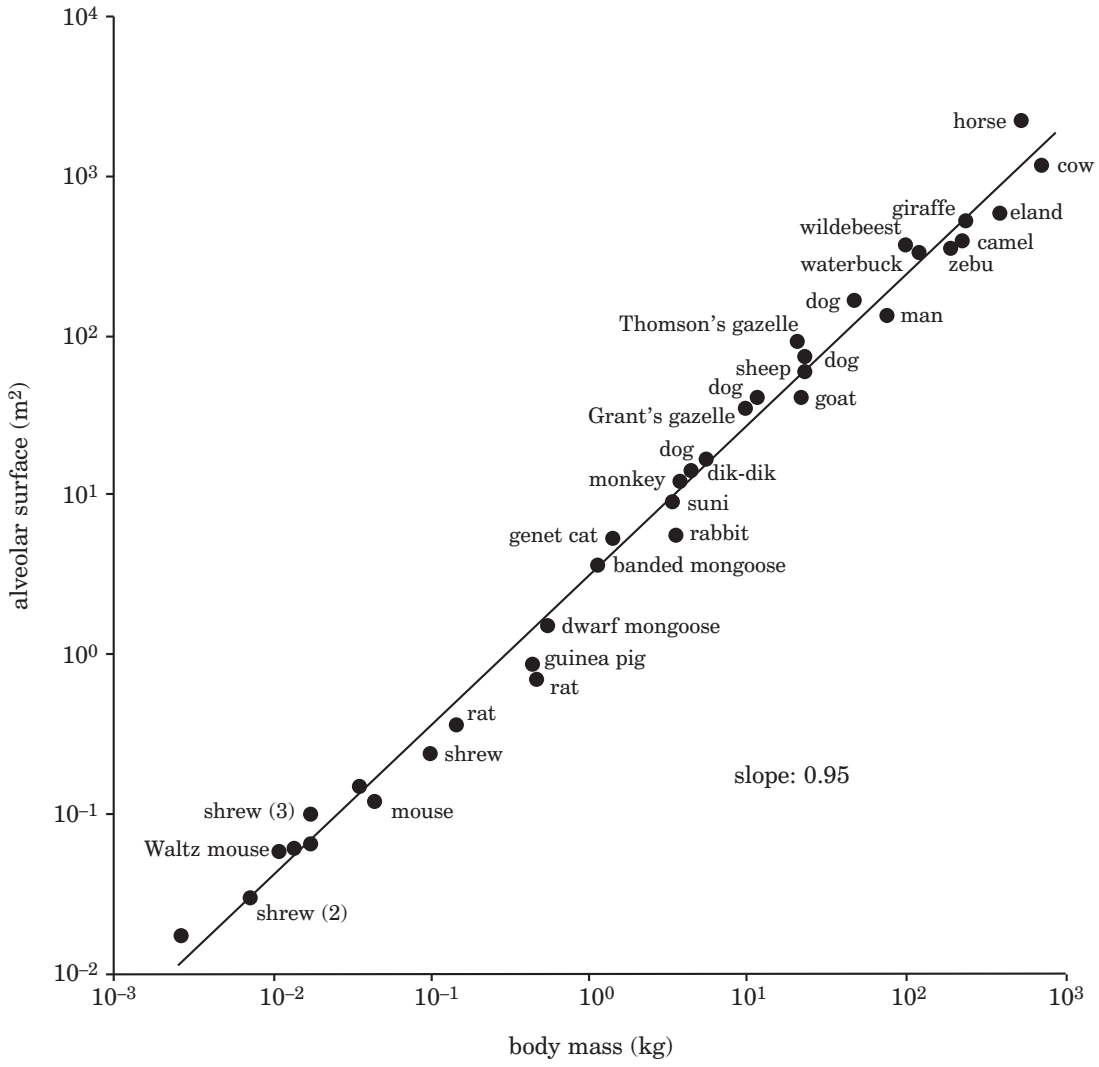
Perhaps the greatest species difference to consider within the mammalian respiratory system is the transition from conducting airways to gas exchange areas. Many species have respiratory bronchioles to transition from a terminal bronchiole to an alveolar duct, but many do not. Respiratory bronchioles, which occur as alveolar out-pockets in the wall of the airway, are particularly extensive in dogs and ferrets and less so in humans and monkeys. In contrast, mice, rats and horses all have terminal bronchioles that lead directly into an alveolar duct.

Significant species-specific variability is also seen in the size, organisation and supply of blood to the lungs. Even the pulmonary acinus, the basic functional unit of gas exchange that arises from the last conducting airway within the mammalian lung, can vary in size and thickness by as much as a factor of two in different species with reasonably similar body sizes.

Gas exchange takes place at a very thin air-to-blood tissue barrier. A large surface area for gas exchange is typical for all mammalian species, and the relationship between alveolar surface area to body mass follows similar linear patterns for most mammals, as shown in Figure 2. A similar linear relationship exists for capillary volume to body mass across mammals. However, the cellular organisation of the gas exchange area shows variability in abundance, size and organisation of these individual cells among species. In addition, the extracellular matrix and basement membrane components show many species-specific features.

All mammalian species have an epithelium composed of Type I cells, which cover approximately 95% of the surface area, as well as secre-

Figure 2: An allometric plot of alveolar surface to body mass



From Pinkerton, K.E. et al. *(13).*

tory Type II cells, which produce the surfactant to reduce surface tension, and cover about 5% of the surface area in all mammalian species. All of this is linked together by connective tissue and a rich capillary bed that is found within the alveolar septum. A tremendous amount of similarity is seen in the composition of the alveolar septal wall across mammal species — in rats, dogs, humans and monkeys. Endothelial cells of the gas exchange region represent 50% of the total cells that would be harvested from that region. Type I and Type II cells each represent about 10% of the total epithelial cell population of the alveolar septum.

When considering methodologies to examine exposure–response relationships, it is also important to factor in site-specific differences as well as species-specific differences. In one study of monkeys exposed to very low concentrations of tobacco smoke, for example, the degree of cytochrome P450 1A1 activity varied according to airway location. Species differences have also been observed in the timing of the development of mammalian antioxidant enzyme activity. Innervation of the airways is another part of the response to a chemical, or a compound, to consider when moving from *in vivo* to *in vitro* methodologies. To summarise, the key points are:

— There is a significant amount of interspecies diversity in the mammalian respiratory system, as well as tremendous similarities.

— The cellular organisation of the gas exchange area shows variability in abundance, size and organisation of individual cells among species. The extracellular matrix and basement membrane components show many species-specific features.

— Species-specific differences are important considerations when using different mammalian species to look at the respiratory system, and lung disease and development.

Considerations and Challenges for *In Vivo/In Vitro* Correlations *(Günter Oberdörster, University of Rochester)*

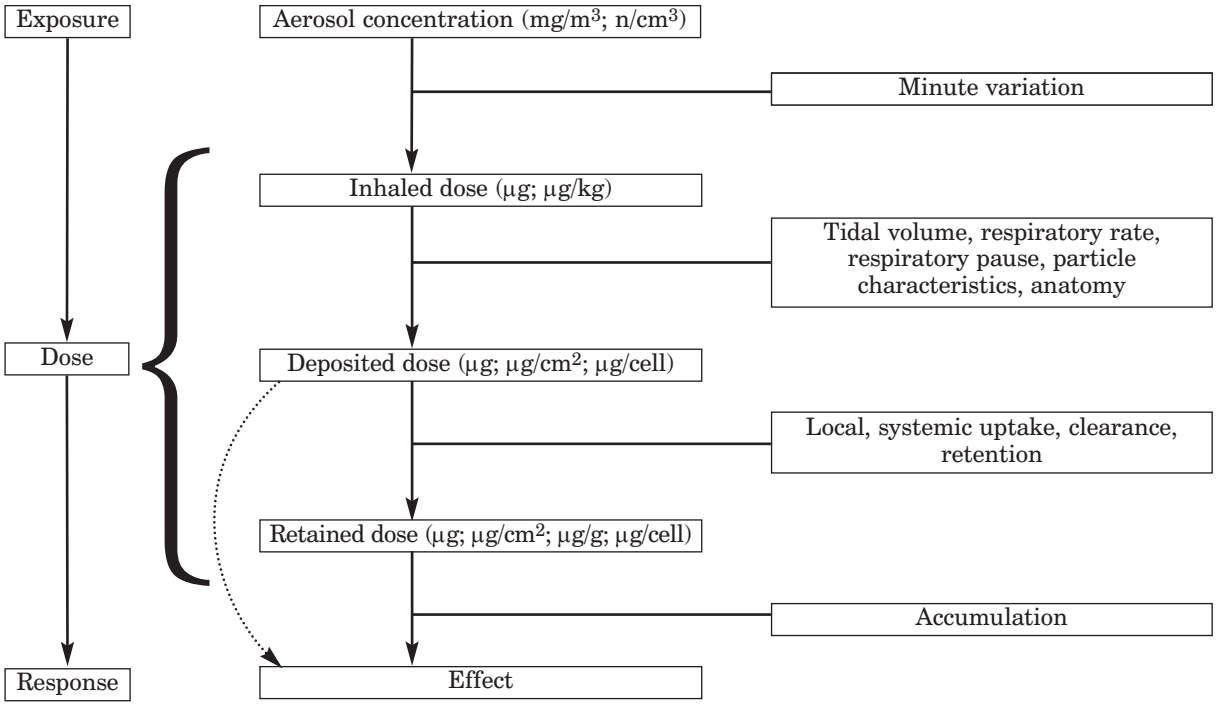
Günter Oberdörster discussed the challenges involved in correlating *in vitro* and *in vivo* dosimetry, the choice of dose metrics, and the relevancy of doses.

Appropriately designed *in vitro* studies may be well-suited for the first step of risk assessment, i.e. hazard identification. However, they have not yet achieved suitability for the final step, i.e. risk characterisation.

Risk assessment is a complex function of both hazard and exposure (14). Exposure is a key consideration and its relationship to dose–response is of central importance for assessing the toxicology of inhaled nanoparticles. Diverse systems for exposure are available including *in vivo* exposures, which tend to be dynamic and used for acute to chronic studies; and *in vitro* exposures, which use diverse cell types, are mostly static systems, and are used for acute exposures. Microfluidic systems (organs-on-a-chip) deal with possibly dynamic systems, but are also basically static. Cell-free systems explore the reactivity of ultrafine particles, with surface reactivity as the dose-metric. Examples include DTT (dithiothreitol) and DCFH-DA (2′-7′ dichlorofluorescein-diacetate) assays, which assess reactive oxygen species (ROS)-inducing potential. These cell-free exposures can be useful as screening tools for hazard ranking. In one study, a comparison of *in vitro* cell-free oxidant activity to *in vivo* inflammatory responses in rats showed a good correlation, suggesting that a simple assay might provide initial information about reactivity (15).

The mechanism of toxicity depends highly on the dose. However, a major problem with most *in vitro* studies, and some animal studies, is establishing the actual dose delivery. For aerosol delivery, a careful characterisation of the airborne particles (e.g. particle size distribution, concentration, effective aerosol density) is required to determine a deposited dose. The factors involved in respiratory tract dosimetry are summarised in Figure 3.

Figure 3: The factors involved in respiratory tract dosimetry



(Upper and lower respiratory tract, secondary target organs)

Detailed dosimetry models have been developed for evaluating realistic dose delivery both *in vivo* and *in vitro*. *In vivo* models allow the prediction of the deposited dose of an aerosol in certain regions of the respiratory tract of humans and experimental animals. *In vitro*, the dose to cells is assessed in a conventional or air–liquid interface system. However, the extrapolation of doses and the results of the mostly acute *in vitro* studies (static; no clearance) to longer-term exposure in humans remains a major challenge.

One approach is to establish benchmark nanoparticles that are well-characterised toxicologically and against which new nanoparticles can be compared. It is important to do these studies both *in vivo* and *in vitro* in terms of multi-dose studies, ranging from No Observed Adverse Effect Level (NOAEL) to the Maximum Tolerated Dose (MTD) to really get the full range of the dose–exposure relationship.

When comparing toxicity *in vitro* and *in vivo*, the challenge comes in aligning *in vitro* and *in vivo* doses. Concepts to consider for in *vitro/in vivo* dosimetric extrapolations for respiratory tract exposures are summarised in Table 2. A proposal is to express dose per cell surface area (or per cell number) and to consider differentiating between the deposited dose (external) and the 'uptake' dose (internal).

Regarding *in vivo* assays, dosimetric extrapolations are accepted methods to derive Human Equivalent Concentrations (HECs) and also eventually Occupational Exposure Limits (OELs) based on data from rodent inhalation studies. Dosimetric approaches are beginning to be more widely applied to assess the effects of different size particles, including nanomaterials. However, a main concern is animal welfare and consideration of the three Rs (*replacement*, *reduction* and *refinement*).

For *in vitro* assays, many challenges remain. For example, what is the cellular dose equivalency *in vivo*? Are acute static systems with no clearance useful? What is the predictability of the chronic effects? Other considerations are the dose dependency of mechanisms and whether they operate *in vivo*. *In vitro* assays are suitable for toxicity ranking against well-characterised benchmarks, i.e. for hazard identification.

Acellular assays, which use predictive toxicity ranking based on surface area-specific reactivity, seem to be a promising screening tool, but require further validation and standardisation. Dynamic dissolution assays are also promising tools for predicting *in vivo* dissolution rates, but require standardisation of the methods. In summary, the key points are:

— *In vitro* studies can be useful for hazard identification and ranking; however, *in vivo* studies are still currently required for meaningful risk assessment.

— Comparative hazard and risk characterisation against positive and negative benchmarks is a useful approach to categorise new nanomaterials. Benchmark materials need to be toxicologically well-characterised and validated, and ideally also certified as reference materials.

— Future goals may include the development of validated alternative simple testing strategies for risk assessment, for efficient, low cost, high-throughput applications.

Table 2: Concepts to consider in *in vitro–in vivo* dosimetric extrapolations for respiratory tract exposures

***In vitro* dosimetry**	***In vivo* dosimetry**
In Vitro Sedimentation, Diffusion Dosimetry (ISDD) model (cells)	Multiple Path Particle Density (MPPD) model (rodent, human)
Physicochemical characterisation Media characterisation	Physicochemical characterisation Respiratory parameters Airway geometry
Diffusion, sedimentation	Diffusion, sedimentation, impaction, interception, charge
Deposition rate (size dependent) and deposited dose (external)	Deposited dose: upper, lower respiratory tract (size dependent)
Uptake: retained dose (internal)	Uptake and translocation to extrapulmonary sites
Clearance: none; static dissolution	Clearance to GI, interstitium, blood, lymph, secondary organs; dynamic dissolution
Retained dose per cell surface area	

Core Subject 1: Tobacco Smoke and E-cigarette Aerosols

Now You See It — Now You Don't: The Chemistry of Cigarette Smoke and E-cigarette Aerosols *(Chris Wright, British American Tobacco)*

Chris Wright described the physical and chemical properties of mainstream cigarette smoke and e-cigarette aerosols, compared their similarities and differences in the context of *in vitro* exposure systems, and discussed the technical challenges associated with their chemical characterisation.

Cigarette smoke is a complex, dynamic respirable aerosol formed by combustion, pyrolysis and distillation, which generate volatile precursors. Oxidative reactions occur as well as heterogeneous nucleation (the process by which particles draw together and increase in size) to form particles with changing size. In comparison, an e-cigarette contains a liquid that is transferred to a coil, which is then heated by a battery. The liquid evaporates rapidly and homogeneous nucleation occurs to form small particles/droplets. Some atomisation or cavitation of those liquids is also seen. In cigarettes, nicotine is largely associated with the tar components; in aerosols, nicotine is associated with the droplets of the aerosol.

Understanding particle size and particle distribution helps us understand the behaviour of cigarette smoke and e-cigarette aerosols in a number of physical and biological systems. It also gives some insight into what might happen chemically. Particle size is probably the single most important parameter to determine for an aerosol, and can be measured by using electrical mobility or laser diffraction. In cigarette smoke, the particle size essentially increases with each puff. This correlates with the fact that as the cigarette rod gets shorter, the tobacco is burned away and the particles coagulate as they are drawn through. So, cigarette smoke is not particularly constant across puffs, and significant differences occur even within the use of a single cigarette. In comparison, an e-cigarette gives a fairly stable particle size distribution, even after extended operating periods.

In a physical sense, cigarettes and e-aerosols are alike in some ways, as summarised in Table 3. They contain similar particle sizes and number. However, a comparison of the chemical compositions of each is more complex. In cigarette smoke, the particulate material is seen in small amounts overall. The bulk of the aerosol is formed by the mass of the air that forms it. In e-cigarettes, the mass is driven by the air. There is a very small proportion of this aerosol that is generated that forms these liquid droplets.

Cigarette smoke contains thousands of substances at varying levels. However, the mass of the organic components of the e-cigarette is largely dominated by three components (propylene glycol, glycerol and nicotine). So, chemical comparisons of cigarettes and e-cigarettes are very challenging. Two-dimensional gas chromatography (GC × GC) has revealed the complexity of the organics found in cigarette smoke compared to those in e-cigarette aerosols, as illustrated in Figure 4. Most cigarette toxicants were not detected in e-cigarette aerosols. Of those detected or quantified, a large proportion was attributable to laboratory air. Such low abundances in e-cigarette aerosol present significant technical challenges to measurement, as well as to assurance of a clean chemical background.

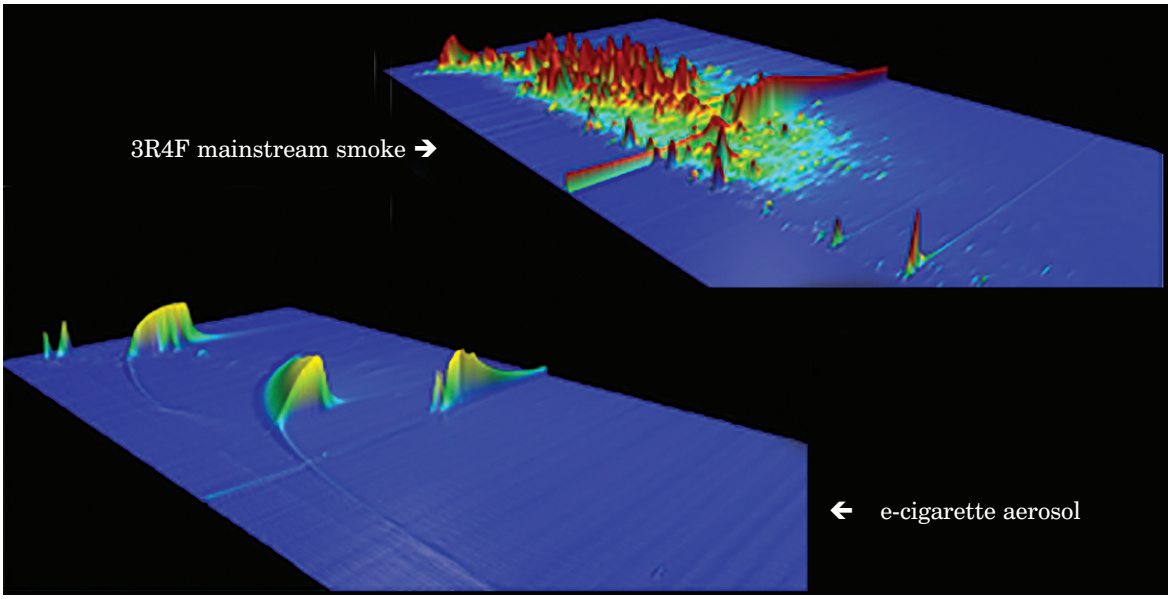
Physicochemical characteristics present challenges, not only in terms of understanding what is happening in smoke and in aerosols, but also what is happening *in vitro*. When working with *in vitro* systems, we tend to be working against dilution, or working with relatively small samples. Other factors

Table 3: A comparison of cigarette smoke and e-aerosol particles

Parameter	Cigarette smoke	e-aerosol
Particle mean size	138–180nm (CMD)	200–500nm (CMD)
Aerosol particle number/cm^3	1,000,000,000 (Ingebrethsen *et al.* [16])	1,000,000,000
Mass per puff (mg)	Ca. 43.3 (ISO)[a]	Ca. 97.8 (3 second, 80cm^3)
TPM/ACM per puff (mg)	1.2 (ISO)[a]	1.5–5.0 (3 second, 80cm^3)
Nicotine per puff (μg)	85 (ISO)[a]	30–100
Puffs per pack	Ca. 180 (ISO, 20 sticks)[a]	200–300 (Cartomizer)

[a]*3R4F (9.4mg tar/0.7mg nicotine/12mg CO).*

Figure 4: The chemical complexity of smoke *versus* that of e-aerosol



GC × GC — TOF-MS analysis of a single 55ml puff.

affecting the transfer of aerosol constituents to cell systems include humidity, coagulation impaction, deposition, diffusion and dissolution. In an aqueous-based system, solubility plays a role in determining what enters the system and how quickly.

Approaches to dosimetry include physical measurement of deposition (by using a quartz crystal microbalance [QCM]) and measurement of markers such as nicotine to quantify aerosol delivery. Direct measurement of the received dose presents significant challenges in terms of dilution, small sample size, chemical selectivity and the selection of appropriate substances to measure, particularly for e-aerosols.

Systems designed for cigarette evaluation may not be compatible with the *in vitro* testing of e-aerosols. High aerosol collected mass (ACM), for example, can be problematic for some systems because QCMs do not tolerate high ACM. Other measures of delivery are needed, but will be challenging to develop. Also, it is important to understand the relative impacts of droplet phase and vapour phase-mediated transfer, and to consider that the effects of lung humidity on particle size might not be replicated *in vitro*. The key points are:

— E-aerosols are physically similar to cigarette smoke in some aspects (e.g. size range), but are chemically very different.

— Systems designed for cigarette evaluation might not be compatible with the *in vitro* testing of e-aerosols.

— The very low abundance of many cigarette toxicants is challenging to measure in the aerosol, and even more challenging to measure *in vitro*.

— Other measures of delivery are needed, but will be challenging to develop.

Core Subject 2: Air–Liquid Interface-*In Vitro* Exposure Systems

Fresh Smoke ALI Exposure in 24 and 96 Multiwell Plates Using a New Smoke Exposure *In Vitro* System (SEIVS) *(Roman Wieczorek, Imperial Tobacco Limited)*

Roman Wieczorek described a newly developed smoke exposure *in vitro* system (SEIVS) in use at the Imperial Tobacco BioLab.

Air–Liquid Interface (ALI) exposures are one of the more recent developments in *in vitro* exposure testing. Due to the dynamic nature of cigarette smoke, a rapid dilution and transport of smoke to the cells is essential. To address this need, Imperial Tobacco BioLab has adapted the SEIVS exposure system, which provides exact dilution and cell exposure in multi-well plates. This system enables *in vitro* testing of aerosols generated from different product categories, including tobacco products as well as e-cigarette devices.

The SEIVS system allows for simultaneous processing of two ALI exposure chambers by using 96 and 24 multi-well plates. For the ALI exposure in the 96-well plate, neutral red-stained HepG2 cells (human liver) were cultivated on collagen I, a proven material for the maintenance of cells and which enables long-term exposure to aerosol under ALI conditions. For the ALI exposure in the 24-well plate, V79 (hamster lung) cells were cultivated on a porous membrane. The smoking procedure simulated natural smoking behaviour (puffing/breathing), with puff-specific distribution of the aerosol over a row of wells. This was accomplished by covering the first row of wells with a sliding lid for the control. After each puff, the next row of wells was covered.

The smoke flow is illustrated in Figure 5. The smoking pumps allow a smoking/puffing of up to five products per run. Separate dilution pumps for each of the two exposure chambers allow parallel testing of whole smoke and the gas/vapour phase, and parallel exposures of cells to different dilution levels and different assays.

The effectiveness of the smoke dilution system and the uniformity of particulate phase deposition in the individual wells were determined by measuring the optical density at 400nm. Reproducible smoke dilution and accuracy of the dilution system were confirmed for both exposure chambers.

When *in vitro* cytotoxicity was measured with the Neutral Red Uptake (NRU) assay, higher cytotoxicity occurred after repeated smoking. With repetitive exposure to the gas vapour phase (GVP) of the CM7 (CORESTA Monitor Test Piece 7) product at selected dilutions, cytotoxicity levels correlated to the amount of active substances delivered to the cells.

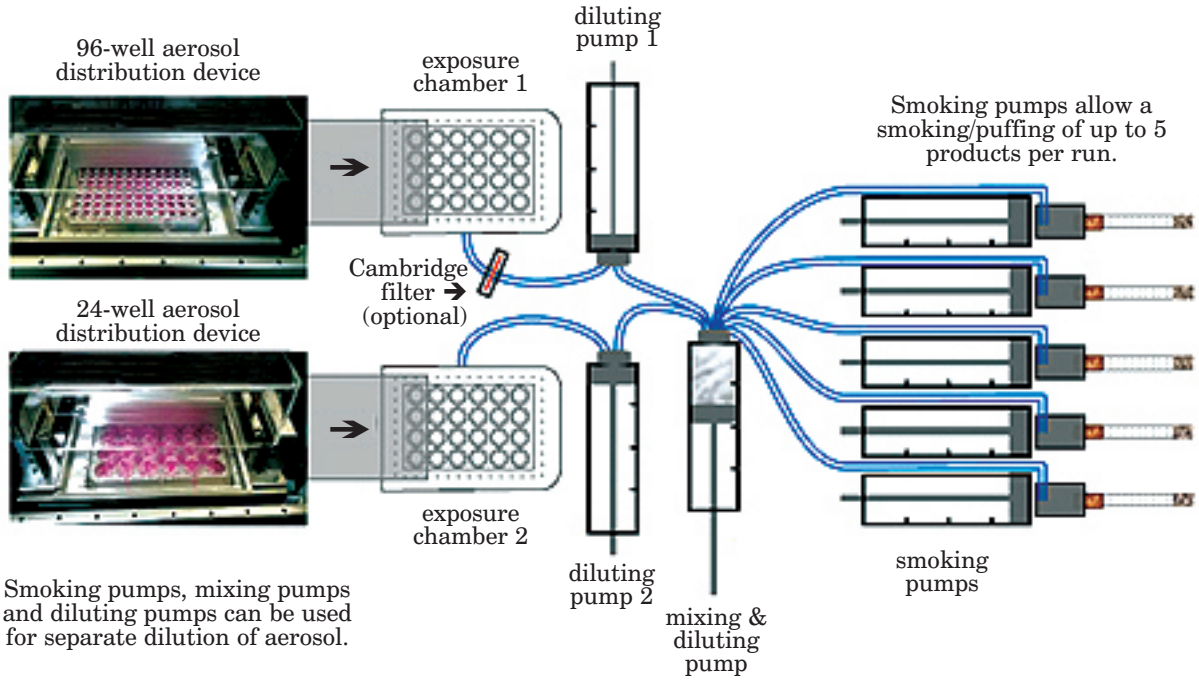
The high sensitivity of the system allows for testing and comparisons of smoke and vapour products. The toxicology of tobacco smoke and vapour was assessed by using four commercial products (CM7, dark blended cigarette, an emerging tobacco product [ETP], and an electronic vapour product) and three assays (NRU [17], *In Vitro* Micronucleus [IVM; 18] and Ames [19]).

The NRU assay after ALI exposures of HepG2 cells showed that the whole smoke of CM7 and dark blend cigarette was more cytotoxic than the emerging tobacco product. GVP contributed significantly to the whole cytotoxicity, so its effects have to be considered.

The IVM assays after ALI exposures of V79 cells showed that whole smoke of CM7 was more genotoxic in comparison to the tested ETP. Vapour of the electronic vapour product did not show any response after 240 puffs. Gas phase components contributed significantly to the whole smoke genotoxicity.

The Ames assay after ALI exposures of *Salmonella typhimurium* bacteria on agar plates showed that the response to substances in GVP correlated strongly with the moisture content of agar in the Petri dishes. Direct bubbling of bacteria suspension guaranteed a fast and effective

Figure 5: Smoke flow in the *Smoke Exposure In Vitro System* (SEIVS)



exposure to all phases of the test aerosol. To summarise, the key points are:

— The SEIVS system delivers reproducible and sensitive results. The short connections and fast dilution minimise the loss of particles before cell exposure.

— The special construction of SEIVS allows testing of up to five products per run, parallel testing of smoke/vapour and their GVP, and parallel exposure of cells in inserts and on collagen I matrix.

— Special features include easy and fast reloading of the exposure chamber by using multi-well plates, and the ability to alternate puffing with smoke and air cleaning, simulating smoking behaviour.

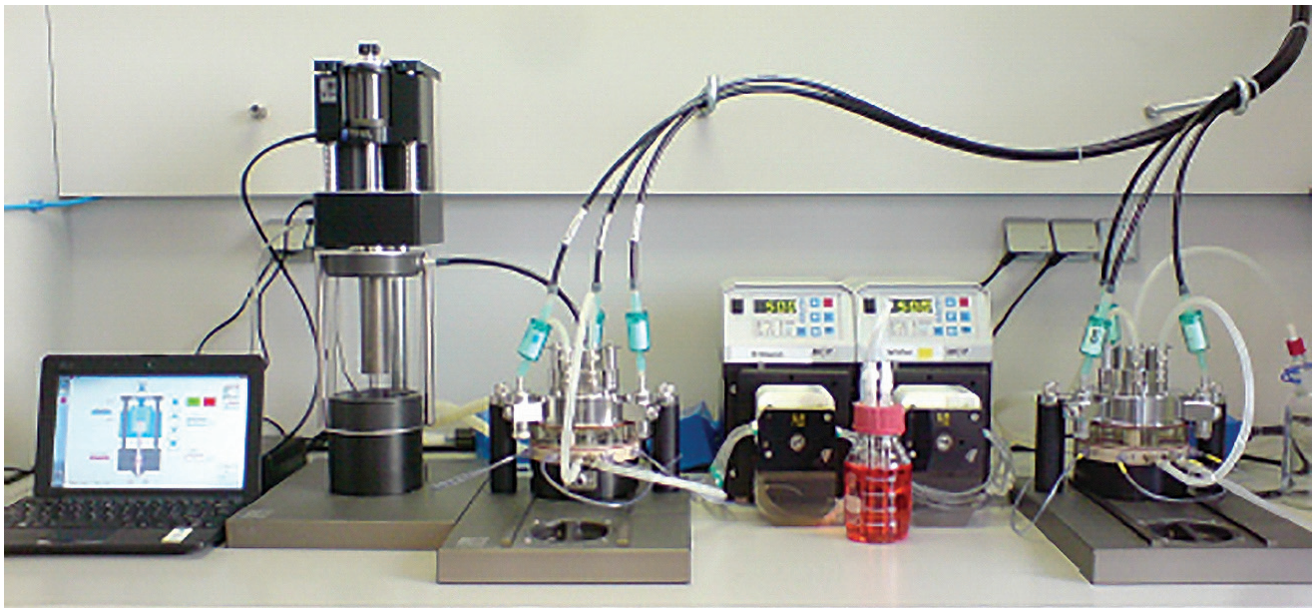
Air–Liquid Interface-*In Vitro* Exposure Systems and Their Use in Inhalation Toxicology *(Michaela Aufderheide, Cultex® Laboratories GmbH)*

Michaela Aufderheide summarised a validation project currently underway to assess the reproducibility of an *in vitro* exposure system used in inhalation toxicology. She also provided an overview of factors to consider when selecting cell-based test strategies for acute and chronic toxicity studies.

Reproducibility is an essential requirement for validating cell-based exposure systems used to study the toxicological effects of inhalable substances. To address this issue, a collaborative validation project funded by the German Federal Ministry of Education and Research is currently underway. Its goal is to establish an experimental protocol for analysing airborne material under standardised, stable and reproducible conditions in cellular-based systems. The project is based on the CULTEX® RFS exposure system, which allows for direct exposure of bronchial epithelial cells at the Air–Liquid Interface (ALI) and the analysis of particulate effects. The system consists of two main parts: the aerosol-guiding module, which conducts and distributes the particles to be deposited on the cell culture inserts, and the sampling module, which has three cell culture inserts or Petri dishes that can be supplied with medium separately. A rack system is also included as a transport and loading platform.

The first phase of the project has been completed (20). Human lung epithelial cells (A549 cells) were exposed to different concentrations of copper (II) oxide nano and microscale particles at the ALI, and cell viability was measured with the WST-1 assay as a parameter of toxicity 24 hours after exposure. The experimental setup consisted of a particle generation unit, the CULTEX DG-Dust Generator, the CULTEX RFS for exposure of the cells to the test aerosols and the clean air control, and a medium supply via peristaltic pumps (see Figure 6). The *in vitro* data showed good overall agreement with existing *in vivo* data for physiological exposures that assessed acute pulmonary tox-

Figure 6: The experimental setup of the CULTEX® RFS exposure system



Aerosol generation | CULTEX® RFS test substance | Medium supply | CULTEX® RFS clean air

icity of airborne materials. This supported the general applicability of the CULTEX RFS with regard to the requirements of the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) on test validity. The project is currently in the second phase, to improve inter-laboratory reproducibility and develop a valid prediction model.

The choice of a cell-based exposure strategy depends on the questions being asked, such as what variables will be analysed, and whether the study is acute (single exposure) or subacute (repeated exposure). Acute studies evaluate dose–response relationships to analyse the toxic potency of inhalable gases, particles and complex mixtures. Subacute studies estimate cellular changes after repeated exposure to non-toxic concentrations of airborne materials for the induction of phenotypic alterations comparable with the *in vivo* situation.

Other considerations include cell susceptibility (i.e. whether cell susceptibility decreases with progressing cell differentiation), as well as cell type (ciliated cells, goblet cells, Type II cells) and cell location (bronchi, bronchioles and alveoli). To address only acute toxicity, an undifferentiated cell or even a cell line can be used to show threshold and the concentration that damages the cells. Other cell types may be required to show particular effects on the respiratory tract. Cellular systems can be monocultures, which include permanent cell lines (tumour and immortalised cells), finite cells (primary cells), or 2-D and 3-D cultures; or co-cultures, which combine different cell types.

For simulating the *in vivo* situation best, the use of primary human cells and immortalised cell lines exhibiting mucociliary differentiation is favoured and recommended. To study the biological effects of airborne material, these cells can be cultured and exposed as mono-cultures and co-cultures under undifferentiated and differentiated conditions, depending on the susceptibility of the cells.

Normal primary lung epithelial cells can be repeatedly exposed to non-toxic concentrations of the test atmosphere at the ALI, allowing the analysis of mechanistic and long-term effects, such as cilia toxicity, mucus secretion or even the induction of hyperplastic and metaplastic changes. Cilia toxicity is one of the first events seen in smokers over the long-term, and increases the risk for developing chronic lung diseases. In one study, normal bronchial epithelial cells were repeatedly exposed (ten times) to mainstream cigarette smoke (4 K3R4F cigarettes; 21). Cigarette smoke induced dramatic changes in cilia and mucus-producing cells after repeated exposure. However, these cells retained the ability to recover to a certain degree. Repeated exposure of an immortalised cell line (CL-1548) to an e-liquid vapour (without nicotine) also caused cilia aberrations. In another study, repeated exposure (13 times) of normal bronchial epithelial cells to mainstream cigarette resulted in the induction of CK 13 positive cells in several, but not all, donors (22). However, a variety of cell types interact during the exposure phase, so these findings require further detailed study. The key points are:

— There are a variety of strategies for exposing cells at the ALI, starting from acute toxicity studies (dose–response relationships) up to repeated exposure studies at non-toxic doses. The choice will depend on the protocol, and a clear definition of what is to be analysed and demonstrated (e.g. cilia toxicity, metaplastic phenotype).

— A validation study of the CULTEX RFS exposure system showed encouraging results, whereby within the first project phase the intra-laboratory and inter-laboratory reproducibility could be demonstrated. Future work (2nd project phase) is planned to improve the database and to develop a valid prediction model.

A New Fluorescence Based Method for Characterisation of *In Vitro* Aerosol Exposure Systems *(Sandro Steiner, Philip Morris International)*

Sandro Steiner discussed a method to determine aerosol losses and delivery in *in vitro* aerosol exposure systems.

Measuring the delivery and dilution of a test aerosol is essential, in order to meet the specific requirements of the biological test system and to assure the application of relevant doses. *In vitro* exposure studies employing the Vitrocell® 24/48 system have been a major component of assessing the biological impact of cigarette smoke *versus* e-cigarette aerosols at Philip Morris Products S.A. The system has two main parts — a climatic chamber and an exposure module consisting of a dilution/distribution system on top of a cultivation base module where up to 48 cell cultures can be exposed simultaneously to a test aerosol that can be diluted serially. With this system, nasal, bronchial or oral organotypic tissues are exposed at the air–liquid interface during 28 minutes, followed by post-exposure times of 4, 24, 48 and 72 hours. Before reaching the tissue cultures, the test aerosols are heated to 37°C, humidified and diluted. Endpoints include cytotoxicity (AK assay), mRNA microarray, pro-inflammatory mediators, ciliary beating frequency and histology.

Although the Vitrocell 24/48 system is highly versatile for delivering undiluted, as well as diluted aerosols, such systems have potential limitations with regard to aerosol losses, as a result of

sedimentation, impaction and anisokinetic particle sampling, which may hamper exact dosing. To determine the suitability of an exposure system for a specific application and its dosing behaviour, aerosol-specific characterisation of the exposure system is therefore required. Such a characterisation aims at obtaining a detailed description of a system's dosing accuracy and precision, its delivery uniformity and reproducibility, and the chemical composition and particle size distribution of the delivered aerosol.

Researchers at Philip Morris International have developed a fluorescence-based method to determine aerosol delivery in *in vitro* aerosol exposure systems. The focus was mainly on the particulate fraction of liquid aerosols, which are becoming more important in the new generation tobacco products. The goal was to develop a direct, robust and fast method for quantification of aerosol deposition at any internal part of the system. For this purpose, model aerosols were generated in a condensation monodisperse aerosol generator (CMAG). The CMAG was chosen because: i) it allows the generation of aerosols of different mean particle sizes and narrow size distributions, which makes investigating particle size-specific effects possible; and ii) a fluorescent label can be incorporated into the particles during their generation. Glycerol, as one of the key components of the aerosols generated by new generation tobacco products, was chosen as aerosol material. As a fluorescent label, the fluorophore disodium fluorescein was chosen because of its high fluorescent activity, stability, water solubility and non-toxicity.

Upon test exposures, deposited aerosol material can be quantitatively eluted from internal system surfaces by using aqueous solvents and, based on the aerosol fluorescence determined beforehand, aerosol deposition in the exposure system can be quantified with high sensitivity and precision simply by measuring the retrieved fluorescent activity in the eluates.

With this methodology, the researchers are currently characterising the Vitrocell 24/48 aerosol exposure system to obtain a detailed description of exposures in terms of reproducibility, deposition uniformity, dilution/mixing effects, aerosol losses, and optimisation of system operation. The methodology may also be applicable for the investigation of aerosol delivery in other cell culture exposure systems. To summarise, the key points are:

— The fluorescence-based method for the characterisation of *in vitro* aerosol exposure systems is a valuable tool to study particle dynamics/delivery.

— Robust particle size-specific generation of disodium fluorescein-labelled glycerol aerosols is possible, as well as robust, fast and sensitive quantification of aerosol deposition.

Cutting-edge *In Vitro* Exposure Technologies for Conventional and E-cigarettes *(Tobias Krebs, Vitrocell® Systems GmbH)*

Tobias Krebs discussed the major components of a typical *in vitro* exposure system for assessing conventional and e-cigarettes — smoke/vapour generation, dilution systems, exposure systems, auxiliary equipment, and dosimetry tools — and the importance of matching all components to the process requirements.

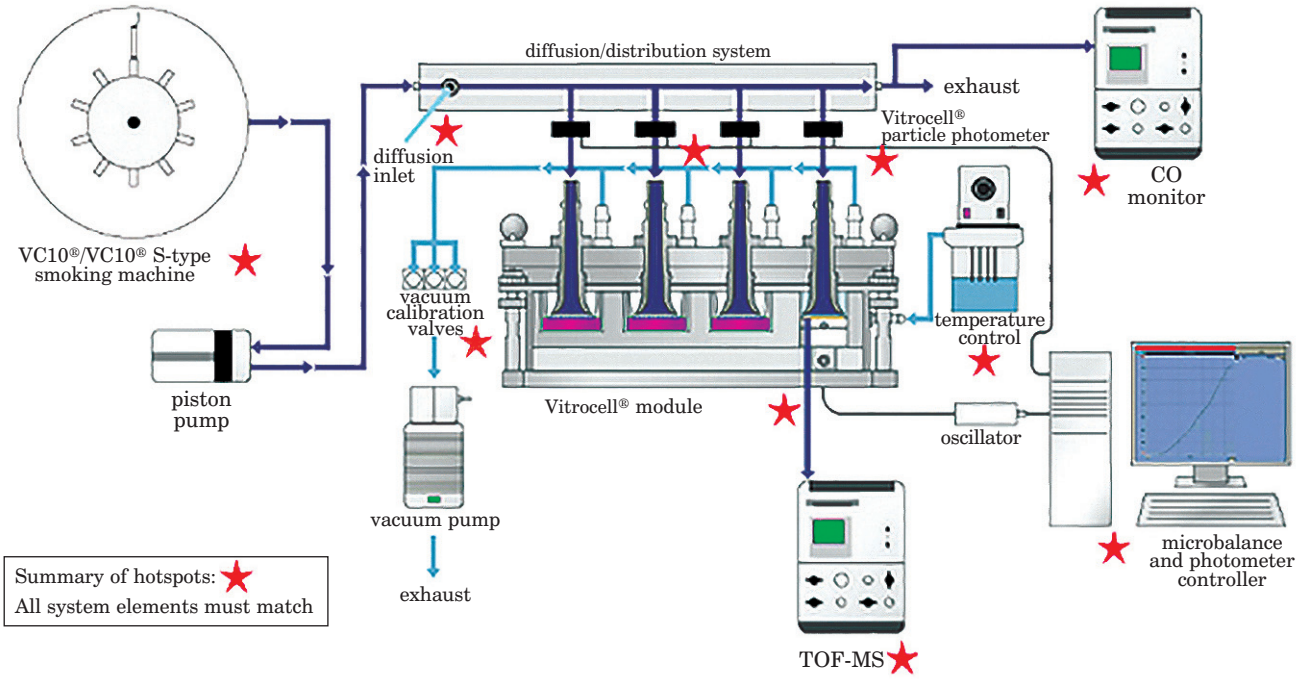
A complete exposure system for conventional cigarettes and e-cigarettes is complex, as illustrated in Figure 7. Critical system elements, the 'hot spots' for *in vitro* exposures, and Vitrocell® product examples are described below:

Smoke/Vapour Generation: This is the first hot spot in an *in vitro* installation. Here, reproducible aerosol generation with the smallest dead volumes is important, as well as a fast and easy cleaning procedure, and the avoidance of cross-contamination when testing products. Smoke/vapour generation differs for conventional *versus* e-cigarettes. For conventional cigarettes, smoking regimens are generally ISO and Health Canada Intense (HCI), with actuation by a lighter. For e-cigarettes, constant flow (square) profiles of 55 or 70ml over 3 seconds with a frequency of 30 seconds are typically used, with the device activated by draw or button. Two types of equipment are available for smoke/vapour generation: automatic robots and manual machines. The VC10 S-type, an automatic robot, has a multi-pump system that can supply various dilution systems by switching from one pump to the other, and can be used in evaluating conventional and e-cigarettes. The manual VC1 smoking machine is more suitable for e-cigarettes, has the smallest dead volumes and offers an increased capacity via multiple pumps. A positive control can be run in the same experiment. Machines can be characterised by analysing the total particulate matter (TPM) and particle concentrations via inline photometers before and after the piston pump, to define particle losses. The VC10 smoking robot is the most characterised smoking machine for *in vitro* applications (23, 24). The VC1 smoking machine was introduced for testing combustion and e-cigarettes (25).

Dilution System: The dilution system is the second hot spot in the system. Here, reproducible dynamic dilutions with the smallest dead volumes are important. Fresh aerosol should arrive quickly to the test system. The system must also be easy to clean. For conventional cigarettes, the typical concentration range is 5–50% smoke. The e-cigarette concentration range is 30–80% aerosol.

Exposure Module: The exposure module must also be reliable and easy to clean, and ensure uniform

Figure 7: The complete Vitrocell® exposure system



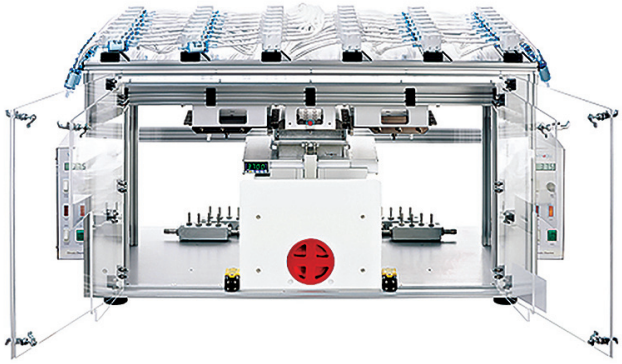
particle deposition. Exposure systems are similar for both conventional and e-cigarettes. Traditional *in vitro* exposure methods, which are still often used, include submerged or suspension cultivation with exposure in an incubator, but have the disadvantage that the test substances interact with the media, and thus give a low sensitivity and an undefined dose. The Air–Liquid Interface (ALI), where cell cultures are exposed on microporous membranes, has the advantage of including all three phases of the aerosol (gas, semi-volatile and particle) in the exposure, giving a high sensitivity and defined dose. This method is more physiologically relevant to the human situation. When selecting an exposure module platform, 3-D must be considered: the membrane insert size, the number of doses or throughput, and the type of assay to perform. When working with bacteria for the Ames assay, for example, the requirements arising from the use of Petri dishes must be taken into account.

Vitrocell offers a wide range of exposure systems for normal and higher throughput that are heavily published. The newest Vitrocell systems are the 6/48 and AMES 48, which meet the demand for higher throughput and a compact design, and that were developed based on the Vitrocell 24/48 technology (26; see Figure 8). The Vitrocell 96 module for 96-well sized cell culture insert plates has an integrated dynamic dilution system, and allows for 11 doses at eight replicates and one clean air control at eight replicates.

Auxiliary Equipment: Auxiliary equipment includes components to optimally manage vacuum flow rates, dilution air flow rates, temperature, humidification and other exposure conditions, and must be synchronised with the exposure modules. Examples include equipment for the maintenance of ISO laboratory conditions for conventional cigarettes and heated chambers for e-cigarettes.

Dosimetry Tools: Finally, dose monitoring is vital for process control and interpretation of the test results. Dosimetry tools for assessing conventional and e-cigarettes are similar, and employ chemical

Figure 8: The Vitrocell® 24/48 exposure system



analysis and time-of-flight mass spectrometry (TOF-MS) for the gas phase. Carbon monoxide (CO) is also measured for conventional cigarettes. In the last few years, advanced solutions for dose assessment became integrated into the exposure systems. These include online TOF-MS technology, which can detect components of the aerosol at a very high resolution. Various concentrations of acetaldehyde and nicotine, for example, can be detected and compared for conventional cigarettes and new generation products.

For assessment of the particle phase, relevant tools are chemical analysis, photometers and microbalance technology. For particle deposition in the *in vitro* exposure system, particle mass and deposition rates need to be evaluated with no disturbance of the exposure process. For this purpose, the microbalance sensor for dose–response measurement was introduced more than five years ago and can be integrated into the exposure systems (27). The sensors are capable of measuring the deposited mass in the module at a resolution of 10ng/cm^2 per second. Inline photometers are used to assess the particle concentration to prove that the product is reproducibly guided to the cell cultures. These enable online measurements of particle concentrations at the inlets and/or outlets of the aerosol exposure top, and can measure at very low flow-rates (e.g. 5ml/min) without any particle losses. Advanced dosimetry software offers a combined view of the photometer and microbalance deposition data, with up to eight microbalance sensors and eight photometers.

In summary, the selection of suitable components depends on many factors, including the nature of the aerosol, the type of assay, sample size, and throughput requirements. All component groups can be tailored to match the needs of the research laboratory or specialised testing facility. The key points are:

— The quality of aerosol generation is vital for a successful *in vitro* experiment. The smoking machine requirements for conventional and e-cigarettes are different, but share the same basic technologies.

— Reproducible dynamic dilutions with the smallest dead volumes are important.

— Sample size and amount of the different doses to be assessed in one experiment are factors to consider in selecting machine type, dilution systems and exposure modules.

— The *in vitro* exposure system should be capable of handling exposure at the ALI.

— Dose monitoring is vital for process control and interpretation of the test results.

Core Subject 3A: Dosimetry Approaches for Particles and Vapours

Modeling Inhalation and Deposition of Cigarette Puff Mixture in the Lungs of Smokers *(Bahman Asgharian, Applied Research Associates)*

Bahman Asgharian discussed the development and application of a mathematical dosimetry model for determining the deposition of cigarette smoke particles in the oral cavity and the lung.

A realistic assessment of the deposited dose of inhaled cigarette smoke in the respiratory tract must address the underlying physicochemical properties of the smoke. Cigarette smoke mixtures have properties that make their behaviour much more complex than that of environmental aerosols. A cigarette puff contains many different chemical components in particulate and gaseous forms. These compounds then enter the respiratory tract after puff withdrawal, are inhaled deep into the lung, and are deposited preferentially on airway surfaces based on their aerodynamic and thermodynamic properties. In addition, the breathing manoeuvre during smoking differs from normal breathing, and contributes to discrepancies in predicting particle deposition. During smoking, the puff is drawn into the oral cavity and this is followed by a mouth-hold. Smoke-free air is then inhaled and mixed with the puff before delivery of the particle mixture to the lung. Particle size may change during the puff drawing, mouth-hold and delivery into the lung.

When freshly generated, most components of cigarette smoke are condensed into droplets. However, there will be a continuous gas–particle conversion (phase change) of components depending on their saturation vapour pressure, deposition on airway surfaces determined by their aerodynamic properties, and coagulation of cigarette particles due to their high number concentration. Particle size is also affected by relative humidity, with increasing size at high relative humidity (as particles absorb water vapour from the surrounding air). Hygroscopic growth occurs quickly and is mainly responsible for particle growth over size increase due to phase change of other components.

Understanding the fate of these compounds in the lung and the localised dose to the lung of the inhaled smoke is essential for studying the health impact from cigarette smoking. Two other major mechanisms affect cigarette particle behaviour and deposition. One is the colligative (or cloud) effects, which occur when a mass of particles behaves as a single body, resulting in airflow moving around the body rather than through it. The

other mechanism involves non-colligative effects, which include phase change (evaporation or condensation of semi-volatile components) and coagulation (movement and collision of particles in the air due to their thermal energy). These mechanisms affect deposition of smoke particles by sedimentation, impaction and Brownian diffusion.

Mathematical dosimetry modelling offers a realistic approach to studying the fate of inhaled smoke and is complementary to controlled studies of biological responses. Modelling efforts begin with studying the behaviour of the puff during inhalation due to droplet–vapour phase change, aerosol coagulation, deposition on airway surfaces, and mixture of the puff with the dilution air at the end of a mouth-hold before entering the deep lung. In developing a model, the assumptions include the exposure parameters (concentration, size distribution, etc.), lung geometry, and breathing rates and profile. The goal is to calculate the deposition fraction of cigarette particles in the lung during a single puff inhalation and during multiple breaths. Challenges include the lung geometry, which is very complex with its varying airway dimensions and branching, as well as the calculation of lung ventilation — the airflow distribution that determines where the particles end up in the lung.

The model presented assumes a simplified airway geometry — i.e. cylindrical airways and a dichotomous branching structure — and uniform expansion and contraction of the lung lobes (28). Particle transport modelling is based on a mass balance equation per airway to calculate the deposition fraction in all lung airways. Calculations of particle deposition for a smoking scenario are based on a simulation of the breathing pattern of a smoker — from drawing of the puff to mouth-hold, inhalation of dilution air, pause, and exhalation.

Model predictions of deposition fractions with and without the cloud effects were compared. The results showed that the cloud effect was most significant in the large airways of the lung and that the effect decreased distally with lung depth. Deposition in the tracheobronchial region suggested a strong cloud effect, while deposition in the pulmonary region suggested a diminishing cloud breakup effect in the deep lung. There was little or no cloud effect in the alveolar region.

Mixing of the puff with dilution air reduced the cloud effect and tracheobronchial deposition. With no mixing, there was significant deposition in the oral cavity and tracheobronchial region due to the cloud effect. With complete mixing, there was a high deposition in the oral cavity due to the cloud effect and a reduced deposition of cigarette particles in the tracheobronchial and alveolar regions. In summary, the key points are:

— Mathematical dosimetry modelling offers a realistic approach for studying the fate of inhaled smoke and provides a link between exposure characteristics and biological responses.
— Consideration of the cloud effect is needed for realistic predictions of particle deposition. Predicted particle deposition considering the cloud effect was greater than when treated as a collection of non-interacting particles.

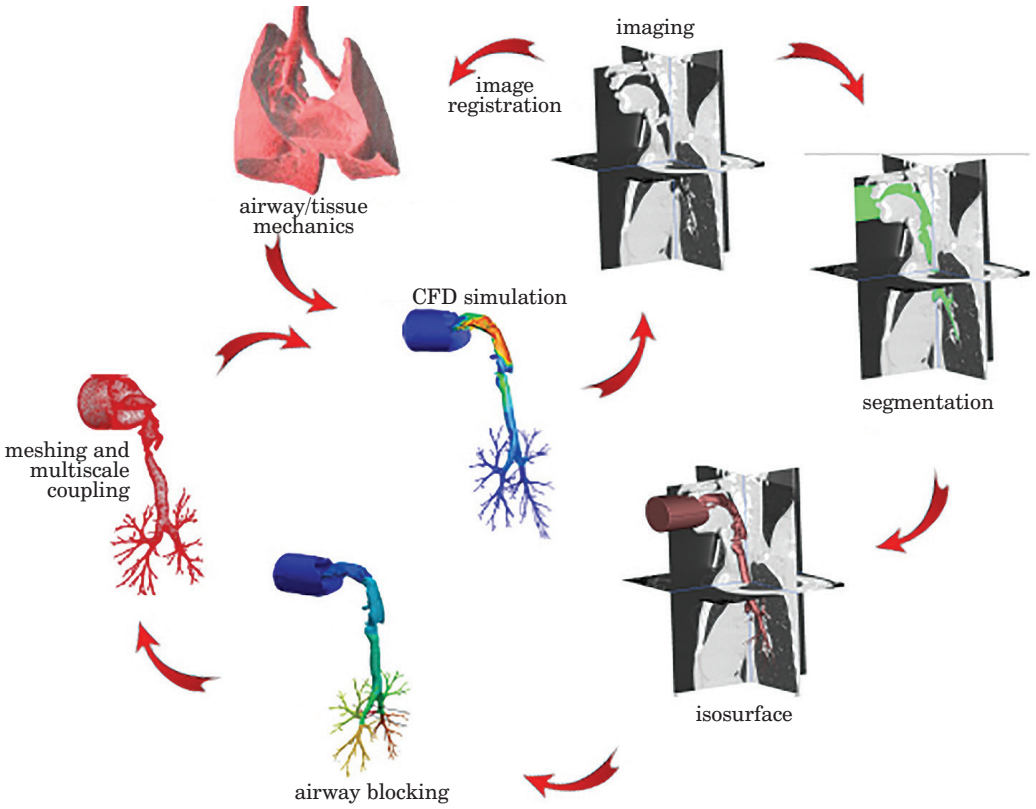
CFD Modelling of Aerosols and Vapours for Cross-Species and IVIVE Respiratory Dosimetry *(Richard Corley, Pacific Northwest National Laboratory)*

Richard Corley used data obtained from computational fluid dynamic (CFD) modelling to address species differences in site-specific aerosol deposition, as well as tissue doses, for reactive aldehyde vapour constituents found in tobacco products under realistic exposure conditions. These CFD models and others serve as the foundation for relating *in vitro* responses to realistic human exposure conditions.

Tools for developing CFD models have evolved considerably since the 1990s, and models can now be developed in days or even hours, depending on the species. The development of an imaging-based CFD respiratory model, which is summarised in Figure 9, begins with magnetic resonance imaging (MRI) and computerised tomography (CT) of the airway features of interest, followed by segmentation of the imaging data and the creation of an isosurface for mapping cell types and tissue types. Computational meshing and multiscale coupling are then employed for CFD simulation. A suite of imaging-based CFD models are now available for a variety of species — including rats, mice, rabbits, monkeys and humans — and personalised models are on the horizon.

In a recent study, extended airway CFD models of the rat and human were coupled with airway region-specific physiologically based pharmacokinetic (PBPK) tissue models to describe the kinetics of three aldehydes found in cigarette smoke: acrolein, acetaldehyde and formaldehyde (29, 30). Aldehydes are highly reactive, water-soluble vapours, and difficult to measure in tissues because of contact site irritation, inflammation, degeneration and mutations. To date, human health risk assessments have been driven largely by cytotoxicity and tumours in the nasal tissues of rats (nose-breathers), as opposed to humans (nasal/oral breathers). To compare site-specific airway tissue internal doses between rats and humans under realistic breathing and estimated cigarette yields, tissue 'hot spots' for each aldehyde in each cell type (nose) or region (other airways) were determined along with overall regional area-under-the-curve (AUC). Hot spot AUCs were defined as a function of concentration, surface area

Figure 9: Imaging-based CFD model development



From Corley, R.A. et al. *(29) and Corley R.A.* et al. *(30).*

and depth within a cell type or region, and constituted the top 2.5% of AUCs for all facets in each region.

In prior steady-state rat simulations of aldehyde nasal toxicity, the anterior respiratory nasal epithelial tissues received the greatest initial uptake rates for each aldehyde. However, with the more realistic transient breathing profiles in this study, AUC concentrations were greater in the anterior dorsal olfactory epithelium. Human oral breathing was simulated by measuring puff ventilation profiles and smoke compositions for representative puff concentrations of each of the aldehydes. In the human simulation, oral and laryngeal tissues received the highest local tissue dose. Penetration to pulmonary tissues was greater than that predicted in the rat. Lifetime average daily doses (LADDs) were compared for each aldehyde under realistic cigarette smoking in humans with those produced in the target tissues of rats following sub-chronic inhalation exposures. Based upon LADD comparisons of tissue hot spot AUCs and numbers of cigarettes smoked/day, the order of concern for human exposures was acrolein > formaldehyde > acetaldehyde.

Another ongoing study involves a CFD model developed to compare the deposition of aerosolised *Bacillus anthracis* spores in the respiratory airways of a human with that of the rabbit, a species commonly used in the study of anthrax disease (31). Results showed that regional spore deposition patterns were sensitive to airway geometry and ventilation profiles. Spore deposition in the nose was higher for rabbits than humans and was attributed to structural differences: the rabbit nose is highly turbinated with a very different anatomy and diffusive properties compared to humans. Deposition in the lower conducting airways was higher for humans than rabbits, and attributed to differences between the two species in the bifurcations of the lung.

Looking toward the future, a comprehensive molecular atlas of the late-stage developing lung is currently in development with funding from the National Heart Lung and Blood Institute (NHLBI). Known as Lung-MAP, this open access reference resource is utilising state-of-the-art molecular and imaging technologies to map and annotate the cell types of the developing mouse and human lung. The goal is to fill the knowledge gap in molecular/

cellular events that drive lung development and cell function, and to provide tissues, reagents and data to the medical research community.

Four-dimensional CT imaging is currently being done in animals and shows that the tissue mechanics of the lung are implicit in its motion. Data from a rat model of late-stage chronic obstructive pulmonary disease (COPD) have allowed for the development of ventilation maps and stress/strain relationships.

Work is also underway on multiscale coupling for aerosol deposition. These models involve 3-D CFD descriptions of airways, with the addition of 1-D models in a bi-directional coupling with airway mechanics, airflow, and multiple path particle density (MPPD) models to get a better full description of the airways. These models also consider changes in respiratory behaviours that contribute to target site dosimetry and response. In summary, the key points are:

— Understanding target tissue dosimetry under both experimental and realistic exposure conditions will be just as vital to successful implementation of *in vitro* testing as it has been for cross-species comparisons.

— Simulations with CFD-based models enable the development of more-realistic and relevant human equivalent exposures associated with responses observed in animals as well as *in vitro* organotypic respiratory cell culture systems at an air–liquid interface.

— Benchmarking responses to target site, or tissue dosimetry, significantly improves the ability to prioritise tobacco product constituents of concern and reduces uncertainties in cross-species and *in vitro–in vivo* extrapolations.

Deterministic Dosimetry for Particles and Vapours: Consideration for *In Vitro* Mainstream Tobacco Smoke and E-vapour Product Studies *(Michael Oldham, Altria Client Services, LLC)*

Michael Oldham reviewed mechanisms and factors to consider when determining dosimetry in *in vitro* studies of mainstream tobacco smoke and e-vapour.

The goals of *in vitro* dosimetry studies have evolved from simply knowing the exposure concentration in the culture, to determining the cell exposure concentrations as well as the cell surface dose that causes the response. Now, the goal is to determine the internal cell dose that results in the response, and even to determine the dose at the receptor inside the cell.

Two major mechanisms that determine the deposition of particles in the respiratory tract are Brownian diffusion and sedimentation. Particle transport to cells is calculated by solving simultaneous equations for both of these forces to show how far a particle will travel in one second in still air. These principles were applied in the ISDD (*In Vitro* Sedimentation, Diffusion Dosimetry) model developed by Hinderliter *et al.* (32) to calculate the movement of particles from the media to the bottom of the vessel in submerged cultures (see Figure 10). The simulations factored in gas content, temperature, Avogadro's number, media viscosity, particle radius, gravitational acceleration, particle density, fluid density and total media height.

In developing the ISDD model, some assumptions were made to simplify the calculations. For example, advection and surface area of the sides of the cell culture dish were considered not significant. Particles, either primary or agglomerates, were assumed to be independent and non-interacting, and a uniform particle distribution at initiation of the experiment was assumed.

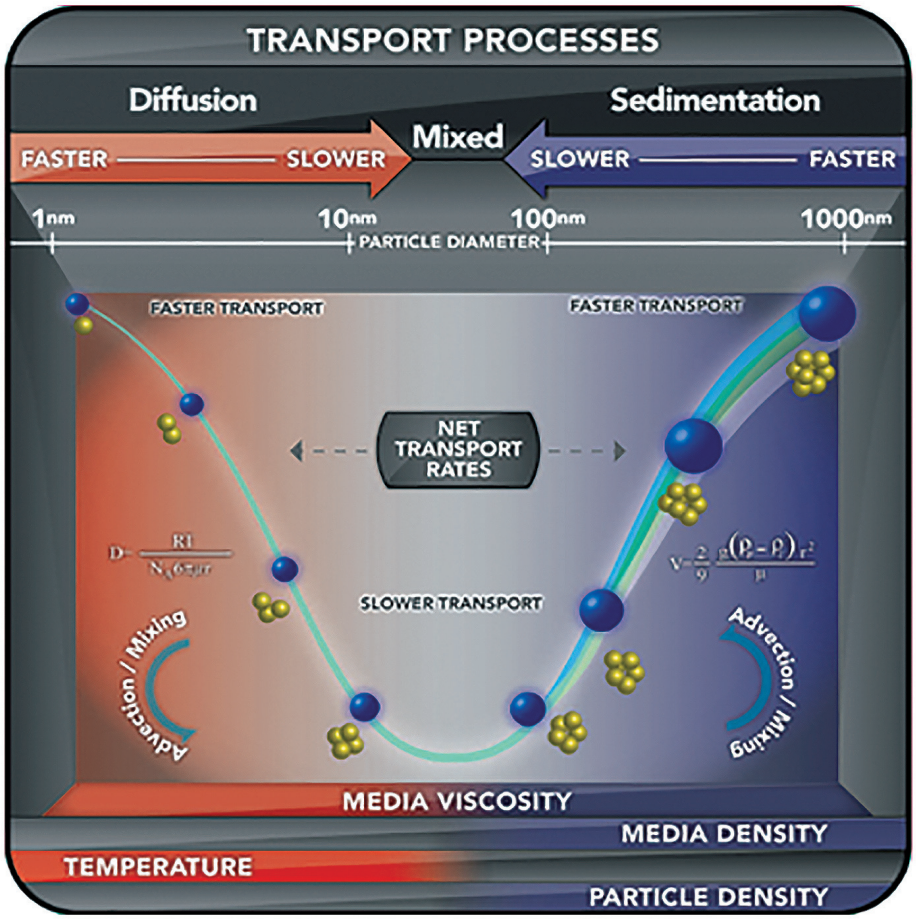
The model was tested against measured transport rates, or cellular doses, for three different particles — carboxylated polystyrene, iron oxide and silica — obtained in three independent studies. Particles of different density, size and agglomeration state were tested. Overall, the cellular doses predicted by the model were in close agreement with the experimental data, differing in most cases by about two-fold. The authors noted that the accuracy of the model was limited by the accuracy of the input data, and by experimental and biological variability. However, the ability to calculate the actual cellular dose ± 50% is an improvement over earlier models.

When using Air–Liquid Interface (ALI) exposure systems, it is important to keep in mind that these systems use low flow rates, which can influence biological effects. Leak detection is critical at every step. These exposure systems have dead space (volume of aerosol transport pathways) and aerosol losses can occur in these spaces prior to cell exposure. External forces, such as thermophoresis created by the temperature difference between the media feeding the cells and the exposure atmosphere, can create a thermal buffer above the cells and affect aerosol deposition (33). Particle charge effects are usually not problematic when dealing with liquid aerosols.

The delivered dose at the ALI can be measured by using a Quartz Crystal Microbalance (QCM). While this works well for tobacco smoke, it has not been effective for the non-Newtonian fluids used in e-liquids. Also, surface area coverage by the aerosol must be considered. For example, are there 'hot spots' in the ALI culture dishes? Are cells in the centre getting more exposure than those in the periphery? More research is needed in this area.

Determination of *in vitro* dosimetry is challenging for both mainstream tobacco smoke and e-vapour product aerosols. Both are concentrated,

Figure 10: Processes and system characteristics affecting particle transport rates in submerged cultures



From Hinderliter, P.M. et al. *(32).*

dynamic and complex, with semi-volatile constituents. Tobacco smoke contains more than 8700 chemicals (34). Dilution of mainstream tobacco smoke can cause changes in particle size, with more semi-volatile constituents in the vapour phase (35). E-vapour aerosols have significantly fewer constituents. However, e-vapour aerosols have a greater proportion of semi-volatile constituents. To summarise, the key points are:

— Knowledge of the delivered dose and its time course is critical to interpreting and potentially extrapolating results from *in vitro* assays.

— Tobacco smoke and e-vapour aerosol present unique challenges, regardless of the exposure techniques used in *in vitro* experiments.

— Quantitative particulate and vapour phase *in vitro* dosimetry determinations are vital to be able to interpret and integrate results of *in vitro* experiments into the scientific literature.

Core Subject 3B: *In Vitro* Dosimetry Determinations

Dosimetry Tools, Approaches and Applications for Tobacco and Next Generation Product Testing *(Jason Adamson, British American Tobacco)*

Jason Adamson discussed the exposure systems and dosimetry tools employed by British American Tobacco (BAT) and how these tools are being used to compare exposure systems and data from cigarettes, e-cigarettes and tobacco heating products.

Only about eight years ago, exposure testing at BAT was mainly focused on combustible cigarettes, which varied by slight differences in factors like rod length, filter and tobacco blend. Now, the next generation products are devices of all shapes

and sizes, with differences in power sources and electronics, and e-liquids with differing flavours/ingredients, nicotine strengths and humectant ratios. Some devices are puff-activated and some require pressing a button. They differ from cigarettes in how they are attached to aerosol-generating machines, how they are held in place, how they generate aerosol, and in their chemical compositions once diluted and deposited *in vitro*. Modes of biological exposure to test article have included:

— *Particulate matter* exposure, which involves submerged exposure to filter-trapped particles that are then washed in solvent. This is a traditional, relatively inexpensive and simple exposure, with much historical data available, based on its use as a regulatory standard. However, only a minority fraction of cigarette smoke can be captured for exposure. Considerations include solvent solubility/interactions, and the physiological relevance for lung cultures.

— *Aqueous extract* exposure, which involves submerged exposure to an aerosol that has been bubbled through media or buffer. This is also a relatively simple and inexpensive exposure that captures both the water-soluble particulate and gas phase components. It is appropriate for most cell culture models and is used, for example, in models of cardiovascular disease and oxidative stress. However, it only captures soluble components, and an analysis of the individual fractions might underestimate the risk. It may be less relevant for lung or Air–Liquid Interface (ALI) cultures. Aerosol solubility and phase distribution must be considered, as well as the type of solvent to use. Some solvents could potentially react with constituents of the smoke fraction.

— *ALI* and *air–agar interface* exposures, which involve whole aerosol or vapour phase only exposure at the air–liquid or air–agar interface. These exposures are more complex and expensive to set up, but are probably the most physiologically relevant for lung cultures and to the consumer, because all fractions and components of the test aerosol are exposed. There is a variety of systems, so individual characterisation is key. It is also important to understand the dilution mechanics, transit, exposure chamber and interface dose of the system, although the differences become less relevant when data can be aligned with dose.

— *E-liquid* exposure, a relatively new method that involves submerged exposure to unaltered e-liquid or its ingredients. It is a very inexpensive, simple and high-throughput exposure. However, one must understand that the components of the e-liquid are changed through aerosolisation. Undiluted e-liquids will be toxic to cells, giving a false positive, but this may not necessarily be an issue, because dose–responses and LD50s can be obtained to make comparisons.

BAT currently works with two ALI exposure systems: the Borgwaldt RM20S® and the associated smoking chamber with a Quartz Crystal Microbalance (QCM), and the Vitrocell® VC10 smoking robot and the associated 6/4 module with QCMs. Five dosimetry methods are used to make product assessments, with two (gravimetric mass and nicotine concentration) employed fairly regularly:

— *Gravimetric mass per puff via QCMs*. The main advantage of this dosimetry method is that it provides real-time data generation, which gives confidence in the exposure. A limitation is that QCMs can be overloaded with aerosol as the droplets coalesce after depositing on the crystal's surface.

— *Nicotine quantification per puff with Ultra Performance Liquid Chromatography (UPLC) Mass Spectrometry (MS)/MS*. The advantage of this method is that it provides relatively quick turnaround in data, and also allows for qualification as well as quantification and conversion of dilutions to delivered nicotine.

Measuring dose at the exposure interface may allow the comparison of data from different exposure systems and products. Table 4 summarises the different methods that have been used to draw comparisons to the wide variety of products currently available. Exposure system dilution provides a very simple data representation, but is only valuable on the same system and does not allow easy cross-platform comparisons. A per stick/product comparison is uninformative, due to the diversity of products with varied uses and delivery. Per puff comparisons may give a closer comparison, but are still limited. A gravimetric mass per puff comparison is a good, real-time, *in situ* quantification of deposited mass of a test article, allowing cross-platform and cross-product comparisons. Delivered nicotine comparisons are even better, allowing *in situ* quantification of a marker across products. Comparisons of other delivered compounds would be best, providing additional *in situ* quantification.

To determine whether dose can be used to align different systems, BAT carried out a case study to assess two cytotoxicity data sets generated on contrasting exposure systems, the Borgwaldt RM20S and the Vitrocell VC10 with different experimental set-ups. Comparisons were made by expressing each data set as a function of dose using $\mu g/cm^2$ and nicotine. The resulting data have been submitted for publication. Overall, the study demonstrated the importance of dosimetry techniques, and how they can be used to align data between two completely different exposure systems and set-ups to facilitate comparisons. The key points are:

Table 4: Methods of dose–response comparison

Method of comparison	Units	Pros/cons and implication
Exposure system dilution	Ratios, %, flow rate	OK; simple data presentation; comparison only valuable on the same system; does not allow easy cross-platform comparisons
Per stick/product	Item	Uninformative; with such a diversity in products that are consumed, puffed, activated in different ways and with varied delivery
Per puff	Number	OK; a closer comparison between products but still limited
Gravimetric mass (per puff)	$\mu g/cm^2$/puff	Good; real-time, *in situ* quantification of test article deposited mass, allows cross-platform and cross-product comparisons
Delivered nicotine	Total ng	Better; *in situ* quantification of delivered nicotine, a cross-product marker
Delivered X, Y, Z	Total ng	Best; additional *in situ* quantification of other delivered compounds

— Dosimetry techniques can be used to align data between two completely different exposure systems and set-ups, to facilitate comparisons.

— Dose tools may provide a link between *in vitro*, *in vivo* and human dosimetry studies and aid in the comparison of data across different tobacco and nicotine product categories.

— The next generation products category will continue to grow, evolve and diversify and dosimetry will support exposure.

Evaluation Method for *In Vitro* Toxicity of Cigarette Smoke by Whole Smoke Exposure *(Xiang Li, Zhengzhou Tobacco Research Institute of CNTC)*

Xiang Li described the whole smoke (WS) exposure system used at the Zhengzhou Tobacco Research Institute (ZTRI), and summarised findings from several assays and *in vitro* dosimetry determinations at the Air–Liquid Interface (ALI).

Cigarette smoke is a complex aerosol composed of thousands of chemicals, which are distributed in a particulate phase and a gas vapour phase (GVP). Investigating only the toxicological effects of total particulate matter (TPM) from mainstream cigarette smoke does not completely reflect the biological effects of the smoke mixture. Direct exposure technology based on the ALI provides a better platform for investigating the *in vitro* toxicity of native cigarette smoke. At present, the representative exposure systems are the CULTEX® and the Vitrocell® systems, as well as the British American Tobacco exposure chamber. These systems provide an ALI exposure for cells and guarantee a composition of mixtures matching the real-life situation.

An experimental platform at ZTRI used a smoking robot VC10 connected with the Vitrocell exposure system. Some parameters, which could potentially influence the measurements, were optimised by using this exposure system. Based on the optimised parameters, *in vitro* toxicity assays with WS exposure at the ALI were established. These included the Neutral Red Uptake (NRU) assay, the Ames assay, and the oxidative stress assay for whole cigarette smoke (36–39).

The results of the NRU assay for WS showed that the viability of cells exposed to synthetic air at a 5ml/min flow rate was not impacted significantly when the exposure time increased. The optimal time point to assess smoke cytotoxicity appeared to be 24 hours after smoke exposure. A good dose–response relationship was observed by using this WS exposure system. The data showed that Chinese hamster ovary (CHO) cells were more sensitive to smoke-induced cytotoxic effects than the human lung adenocarcinoma epithelial cell line (A549 cells). Cytotoxicity under the International Organisation for Standardisation (ISO) regimen was less than that under the Health Canada Intensive (HCI) regimen, when smoke doses were expressed as a percentage of cigarette smoke. Notably, when smoke doses were converted to TPM (μg), cytotoxicity under the HCI regimen was less than that under the ISO regimen. GVP of cigarette smoke plays an important role in toxicological impact.

For the Ames assay for WS, a flow rate of 5ml/min was found to be suitable and a good dose–response relationship was observed with this WS exposure system. A positive response was observed by using the spread culture method rather than the overlay agar method. An S9 mix of 10% was determined to be optimal when considering the positive response and the costs.

The results of the oxidative stress assay showed that WS caused oxidative stress in A549 cells at the ALI. The ratio of reduced glutathione (GSH) to oxidised GSH (GSSG) decreased. Malondialdehyde (MDA), 4-hydroxynonenal (HNE), extracellular superoxide dismutase (ECSOD), and 8-hydroxy-2′-

deoxyguanosine (8-OHdG) levels increased after WS exposure.

Measurements from TPM and WS exposure were compared by converting the EC50 values from WS exposure testing. The comparison was based on the assumption that the TPM of cigarette smoke entering the exposure module could be completely absorbed by the medium during WS exposure. Smoke dose in TPM exposure is generally expressed as μg/ml, while WS exposure is expressed as a percentage of cigarette smoke (% of cig.). The values of % of cig. in WS exposure experiment were converted to TPM-equivalent values by multiplying the % of cig. values by TPM delivery per cigarette. Then, the TPM-equivalent values were divided by the volume of medium in the exposure module. The final results were the values converted to μg/ml.

The results showed that the converted EC50 values in the WS exposure were lower than the EC50 values in the TPM exposure. However, this comparison was based on an assumption and the data conversion has some limitations. For example, the TPM of cigarette smoke might not be completely absorbed by the medium during WS exposure. Also, the converted EC50 values in WS exposure, according to the assumption, might be higher than the actual data. In spite of this limitation, the converted results can indicate that the cytotoxicity of cigarette smoke by WS exposure is greater than that of TPM exposure.

In another experiment, the quantification of deposited particle mass and nicotine on a Quartz Crystal Microbalance (QCM) surface was analysed to assess smoke dosimetry. Nicotine was selected as a chemical marker of smoke dosimetry. The data (unpublished to date) showed a good correlation between the concentration of deposited particle mass and the concentration of nicotine. In summary, the keys points are:

— In the area of *in vitro* toxicity testing based on WS exposure, the dosimetry determination is an important aspect for the ALI exposure experiments.

— Accurate dosimetry data can support the results from *in vitro* toxicity of cigarette smoke.

Core Subject 4: Exposure Microenvironment/Physiology of Cells

Exposing What? Overview of the Airway Tissue Exposure Site *(Holger Behrsing, IIVS)*

Holger Behrsing reviewed the current understanding of human pulmonary structures, the involvement of airway tissue changes and cell types in exposures and responses, and the relationship of these human lung components to two *in vitro/ex vivo* models currently in use for exposure assessments.

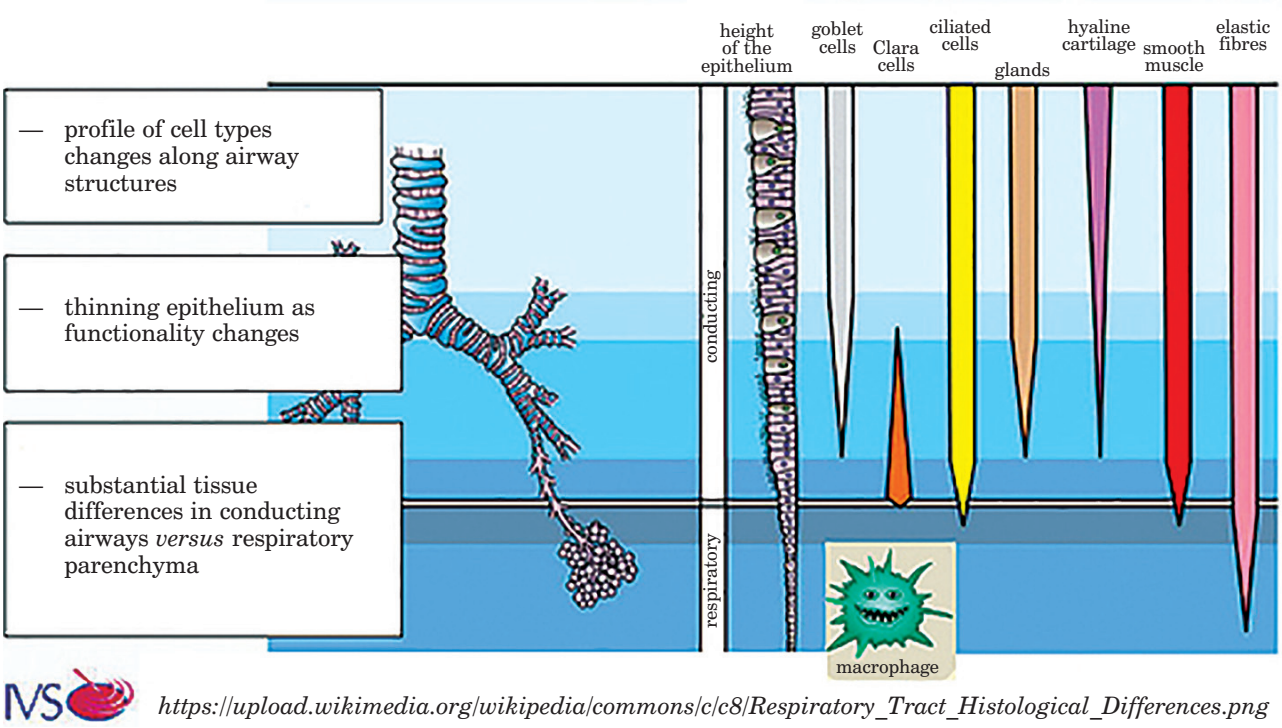
The complex structure of the human lung presents a challenge for quantifying exposures to inhaled materials. In the respiratory tract, cell types and functionality change along the airway structures with substantial tissue differences seen in the conducting airways *versus* the respiratory parenchyma (see Figure 11). Deposition of materials in the lung is also quite variable, depending on the location in the respiratory tract — i.e. the nasal cavity, trachea, bronchus or bronchioles. Different deposition mechanisms, such as diffusion, sedimentation, inertial impaction, interception and electrostatic forces, can play major or minor roles.

The fluid lining of the pulmonary barrier is the first line of defence when a substance enters the lungs. In the upper airway, goblet cells secrete gel-forming mucins, the major components of mucus. The mucous layer contains antiseptic enzymes (such as lysozymes), immunoglobulins, inorganic salts and proteins (such as lactoferrin). In the smaller airways, Clara, or club cells, secrete surfactant and produce enzymes that detoxify substances dissolved in the respiratory fluid. The fluid lining has anti-oxidant properties as well. When quantifying materials at the exposure site, it is important to keep in mind that substances like aerosols and whole smoke will interact with the fluid lining and modify the content of the lung region-specific barrier. The cells and tissue are actually then exposed to a 'modified' liquid, which can be quite variable depending upon the region of the lung.

Various 3-D *in vitro/ex vitro* models are available for assessing exposure responses, including *in vitro* reconstructed human airways (RHuA) and precision-cut lung slices (PCLS), also of human origin. These models are currently being used for studies of cytotoxicity, viability and functional responses (e.g. inflammatory) to exposures. An advantage of the 3-D models over 2-D models is the presence of multiple cell types, including mucus-producing cells. However, while controlled exposures are possible for these models, dosimetry remains a challenge.

The RHuA model allows for airway-like exposures with a number of cell types present, including ciliated columnar cells, goblet cells, basal cells, fibroblast co-culture and club/Clara cells (see Figure 12). Grown at the air–liquid interface, RHuA tissues offer apical and basal compartments that allow flexibility in modelling physiologically relevant exposures, but also allow sampling for location-specific quantification of biological responses. Examples of RHuA models include MatTek's EpiAirway™ and Epithelix's MucilAir™, which can be derived from primary cells of

Figure 11: Overview of the respiratory tract, including airway tissue transitions



https://upload.wikimedia.org/wikipedia/commons/c/c8/Respiratory_Tract_Histological_Differences.png

bronchial origin, but can also be created from cells of the nasopharyngeal region. Newer models include Epithelix's SmallAir™ model, which has a population of Clara cells, and MatTek's EpiAlveolar™ model, which includes epithelial, fibroblast and endothelial layers. A common feature of these RHuA models is that they offer an apical exposure site and they are all grown on a microporous membrane, which allows nutrient delivery from the basolateral compartment containing medium. RHuA models are increasingly being used to assess inhalation exposures, such as cigarette smoke and e-cigarette vapours.

The PCLS model retains the native architecture of the lung and at least some of the common elements that occur *in vivo*. One of the benefits of the slices is the ability to see small areas, like the parenchyma in its native architecture. Also, all of the cells in the tissue are present at slicing, including the macrophages, an important consideration when looking at inflammatory responses. A limitation of this model is that larger airways might be excluded from slicing due to the size constraints of the slicing equipment. In the PCLS model, the cross-section of tissue is exposed, and this differs from an exposure created within the airways and travelling down the airways as it occurs *in vivo*.

When comparing models, it is important to consider the exposure system and whether a vapour, aerosol or smoke is being exposed to the tissue itself. The differential particle/material distribution onto airway tissue regions is variable and dynamic. Questions remain about how to ensure that *in vitro*/*ex vivo* models receive the intended dose. For small airway or alveolar exposures, more research is needed to determine whether it is possible to limit exposure to just the particles/materials that reach the sites *in vivo*. The models are continuing to evolve. To summarise, the key points are:

— Creating accurate and dependable means to quantify pulmonary exposures to inhaled materials, including tobacco-related mixtures and constituents, is challenging due to the complex structure of the human lung.

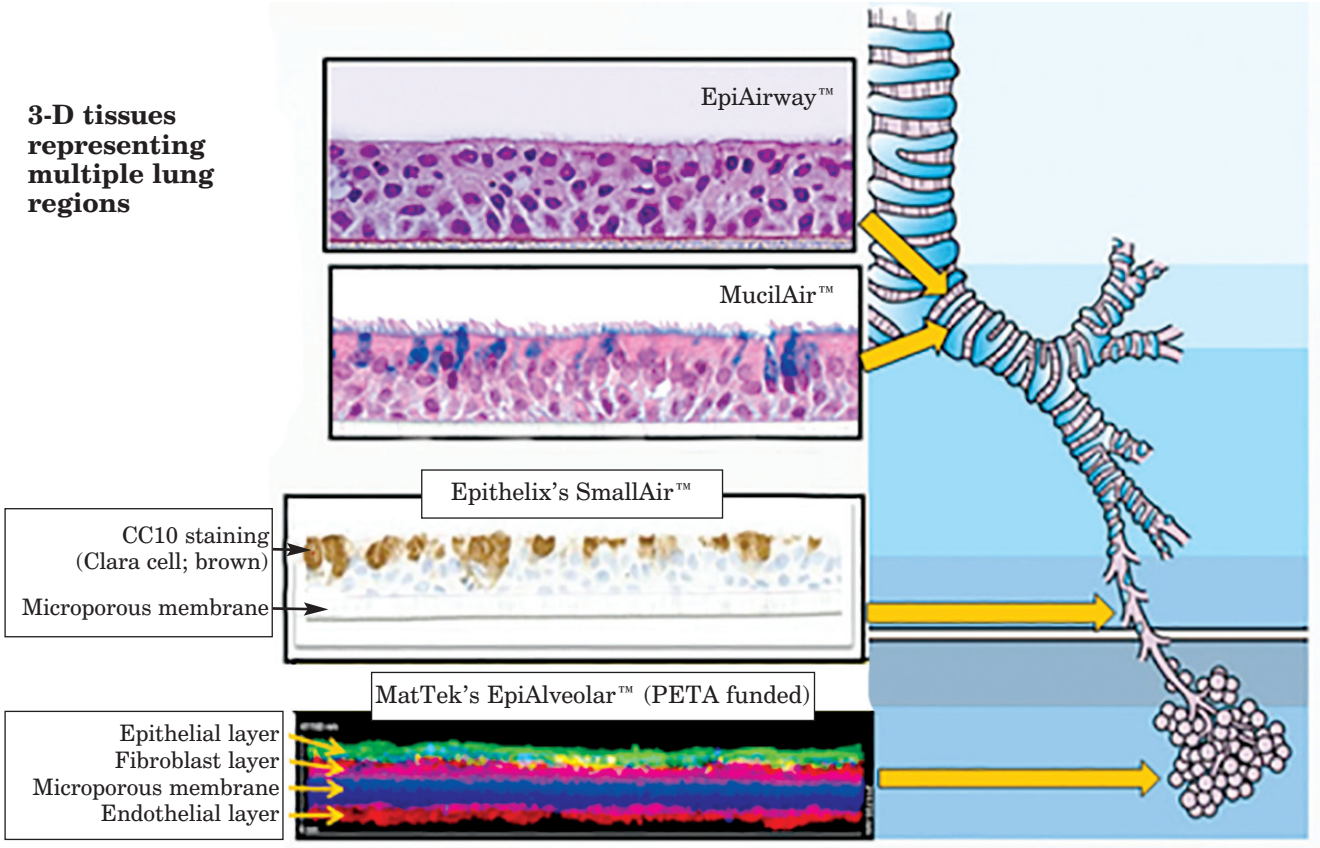
— The use of state-of-the-art *in vitro* tissue models to obtain informative data for correlation back to *in vivo* pulmonary exposures adds yet another factor of complexity.

— A detailed understanding of how these models relate back to native human airway structures and the cells involved in responding to tissue challenge is required.

In Vitro Toxicology of E-cigarettes and Other Tobacco Products *(Irfan Rahman, University of Rochester Medical Center)*

Irfan Rahman highlighted the potential deleterious oxidative and pro-inflammatory effects of e-cigarette aerosols when exposed directly to lung cells.

Figure 12: The reconstructed human airways (RHuA) model



The consumption of electronic cigarettes is rising, particularly among young people. An alarming trend among younger users is the use of a 'dripping' technique, in which the user drips an e-liquid directly onto the e-cigarette's heating coil, rather than into the refillable chamber (see Figure 13). The user then inhales the heated aerosol, which gives a stronger 'hit', as well as the ability to switch between brands, flavours or nicotine content.

It is well known that cigarette smoke and tars contain oxidants/reactive oxygen species (OX/ROS), which mediate inflammation and are implicated in the pathogenesis of lung diseases such as chronic obstructive pulmonary disease (COPD). A recent study, with a modified 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescein-derived dye to detect OX/ROS reactivity in a cell-free system, showed that OX/ROS are generated in e-cigarettes and e-liquids as well (40). One of the sources of the OX/ROS appears to be the activation of the heating element. The amount of OX/ROS reactivity was also dependent on flavour additives; flavours containing sweet or fruit flavours were stronger oxidisers than tobacco flavours. The use of the dripping technique generated an even larger amount of OX/ROS, suggesting that this emerging trend delivers a larger

Figure 13: The 'dripping' technique

dose of OX/ROS to users. Aerosols produced by dripping the e-liquid directly onto the heating element wick resulted in high range DCF fluorescence, as shown in Table 5.

The same study also evaluated cellular toxicity and inflammation in human airway epithelial cells (H292) exposed to e-cigarette aerosols and nicotine. The system employed consisted of an air–liquid interface that utilised a specialised trans-well chamber to mimic pulmonary air–liquid flow dynamics during e-cigarette aerosol exposures. Exposure of these cells resulted in increased secretion of pro-inflammatory cytokines (such as IL-6 and IL-8) into the culture media after the cells were maintained in culture for 16 hours. The H292 cells also showed an increased secretion of IL-8 in response to a cinnamon flavoured e-liquid, suggesting that flavoured e-aerosols, which contribute to encouraging frequent use of e-cigarette aerosols by young people, may cause additional stress and toxicity to the lung tissue. Human lung fibroblasts also exhibited stress and morphological changes in response to treatment with the e-liquids.

The researchers extended this study to a mouse model of e-cigarette aerosol exposure, with wild type (C57BL/6J) mice. The effects of short-term exposure (three days) to e-cigarette aerosols on lung inflammation, oxidative stress and redox physiology were examined by measuring changes in glutathione levels. The results showed that exposure to e-cigarette aerosol increased pro-inflammatory cytokines and decreased the levels of total and oxidised glutathione in the lung cells.

E-cigarette aerosols have also been found to contain copper, a transition metal that can generate even more free radicals (41).

A comparison of conventional cigarettes and e-cigarettes in mediating inflammatory responses will require further experiments in various settings, conditions and cell lines to understand the mechanisms. Studies have been carried out to assess biomarkers of oxidative stress non-invasively in breath condensate (42) and the effects of oxidative stress and cigarette smoke on chromatin histone modification in lung cells (43). The keys points are:

— Tobacco products, such as cigarette smoke and e-cigarette aerosols, are capable of generating reactive oxidants and depleting glutathione in human lung cells and the mouse lung.

— The oxidative reactivity produced by the 'dripping' technique with e-liquids may place consumers at even greater risk for lung damage.

— Differential *in vitro* toxicological testing is possible for different products for hazard ranking based on their chemical constituents.

Promising Technologies

3-D Lung Tissue Constructs, Lung-on-a-Chip and Response to Toxicants *(Sonia Grego, RTI International)*

Sonia Grego reviewed the features of RTI International's 3-D lung-on-a-chip, a biomimetic multicellular model of the airways based on primary human cells, and its potential use for studying responses to drugs and toxicants.

Recently, the development of biologically relevant 3-D models of human tissues has been intense. The goals of this research are to achieve enhanced physiological relevance by controlling topography, biochemical, mechanical and fluid shear stress factors in an engineered microenvironment. These novel models strive to mimic the cell–cell interactions and tissue microarchitecture of the *in vivo* tissue. In addition to the lung model,

Table 5: 'Dripping' technique in refillable e-cigarettes leads to high-range levels of oxidants

	State of the heating element			
Experiment 1	**New**	**2nd use**	**3rd use**	**4th use**
Powered	33.28	8.99	5.68	135.6
Air (sham)	1.60	1.50	1.39	—
Experiment 2	**Pre-used**			
	Clearomiser filled with e-liquid		**Emptied clearomiser with wicked e-liquid**	
Humectant	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**
Consumer refill	47.55	37.42	192.40	250.50

Each value represents the H_2O_2 equivalents (μM) measured after aerosols or clean air is drawn through a dichlorodihydrofluorescein (DCFH) solution.
From Lerner, C.A. et al. *(41).*

engineered cellular models have been developed at RTI to study neuroinflammation and barrier properties in the neurovascular system (44) and drug adverse effects on the heart by using stem cell cardiomyocytes (45).

Lung models have been designed to study the effects of drugs and respiratory virus infections. The lung-on-a-chip model developed at RTI International is a fluidic-enhanced airway model (FEAM) that uses three vertically stacked culture chambers to emulate the microarchitecture of the airway mucosa, as illustrated in Figure 14. The primary human cells used are airway epithelial (AE) cells at the Air–Liquid Interface (ALI), fibroblasts (Fb) to mimic the lung interstitium, and a polarised microvascular endothelial (MvE) cell layer (46).

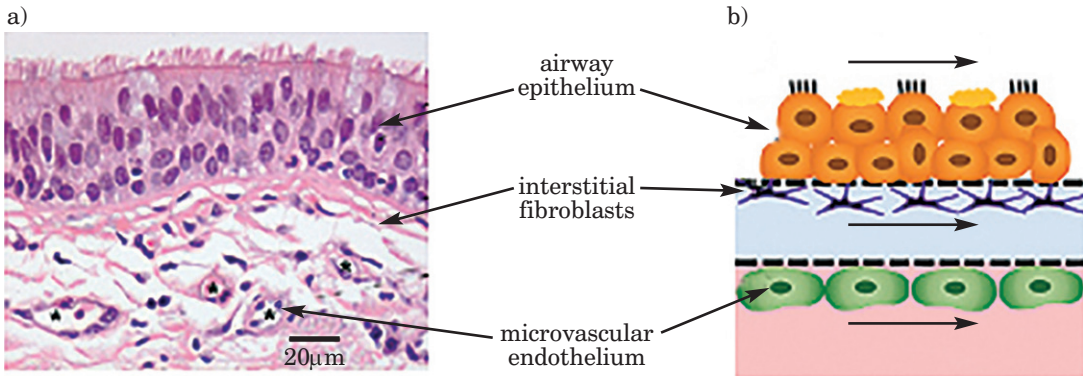
The multi-compartment microfluidic devices are single-use, gas permeable devices, fabricated in optically transparent polydimethylsiloxane (PDMS). Cells are cultured on two nanoporous membranes (0.4μm pore size) that provide support for the AE and MvE cells. A system with vertically stacked cell culture layers is achieved by the three-compartment design, which is obtained by sequential bonding of two different membrane types, as shown in Figure 15. For triple co-cultures, membranes are collagen-coated with different collagens by filling the top, middle and lower compartment with the collagens and allowing them to dry (47). Challenges associated with the devices include dealing with long-term primary cell cultures in microfluidic devices and the fact that each primary cell type prefers a specific medium. Throughput for culture in microfluidic devices is much lower than in conventional static cultures.

Other lung-on-a-chip models include a device developed by Huh *et al.* (48) that features a stretchable membrane and *in situ* mechanical 'respiration'. This microfluidic device reconstitutes the alveolar–capillary interface of the human lung by using the H441 alveolar epithelial cell line and primary MvE cells. Normal breathing patterns are mimicked with channels that experience air and fluid flow and cyclic mechanical strain. PDMS is used as the membrane material.

The presentation included a few slides with an introduction to the features and operation of microfluidic cell cultures. Microfluidic devices are transparent and routinely observed by microscope. Water-tight operation in a humidified environment is sustained long-term (five weeks). Cells at seeding and reagents for assays are introduced with syringes (e.g. needle gauge 22 fits tightly into the tubing and is appropriate for this use). Constant fluid replenishment occurs by passive flow and requires no external power. For specific assays, a faster fluid flow is achieved through active pumping by a small peristaltic pump which fits in an incubator.

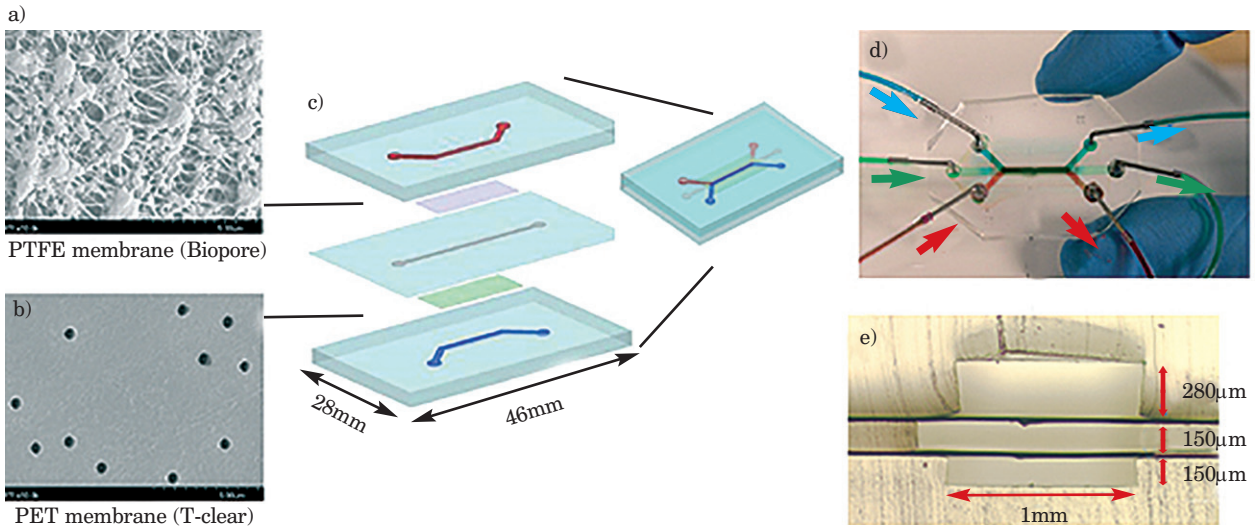
Microfluidic co-culture with all primary airway cells was demonstrated. Six human lungs were processed to obtain primary AE cells, Fb and MvE cells. Experiments with AE cells at the ALI were carried out in parallel in Millicells® and microfluidic devices. A primary AE cell culture in microfluidic devices was achieved with well-differentiated, mucus-secreting and ciliated cells. Airway epithelial cell barrier properties were characterised. Fluorescein isothiocyanate (FITC)-dextran permeability was used for barrier property

Figure 14: The Fluidic-Enhanced Airway Model (FEAM)



a) Histology cross section of normal human bronchus from a lung transplant donor (haematoxylin and eosin stain). Asterisks mark capillaries. b) Schematic of the airway mucosa model, including three vertically stacked compartments with three different cell types separated by two nanoporous membranes, with arrows indicating the channels for fluid or air.
Reproduced from Sellgren, K.L. et al. *(46) with permission from the Royal Society of Chemistry.*

Figure 15: Microfluidic device configuration



a) and b) show scanning electron microscope images of the polytetrafluoroethylene (PTFE) and polyester (PET) membranes; c) schematic view and d) photograph of a 10 × 1mm device with dyes in the three fluidic channels; e) optical microscope image of a 10 × 1mm device cross section.
Reproduced from Sellgren, K.L. et al. *(46) with permission from the Royal Society of Chemistry.*

characterisation. Functional co-cultures were demonstrated first with primary AE and MvE cells in Millicells on day 5. The results showed that AE cells formed a much tighter barrier than MvE cells, and that the permeability of the AE/MvE cell co-culture was dominated by the AE cell component.

A pilot study is being carried out to compare the responses of co-cultures and monocultures to toxic compounds (IL-2, bleomycin) and effective drugs (dexamethasone). All compounds are delivered to the basolateral compartment and added to the medium. Cytokine release and barrier properties will be assayed. The response of the co-cultures in selected cases and endpoints is different from the combination of the response of its components, indicating the effect of cell–cell interactions. In summary, the key points are:

— Microfluidic airway models based on primary human cells in a relevant biomimetic configuration will improve physiological relevance and will enable novel disease modelling and drug development studies.

— The feasibility of the lung-on-a-chip with primary human lung cells has been demonstrated.

— Microfluidic cell cultures feature trade-off between enhanced functionality and throughput.

— Co-culture responses to perturbation differed from that of individual cells, demonstrating that heterotypic cell interactions matter, i.e. 'the whole is different than the sum of the parts'.

Quantitative High-throughput Gene Expression Analysis Using a Modified RASL-seq Platform Enables Treatment-Response Kinetic Analysis for Risk Assessment *(Pei-Hsuan Chu, National Center for Advancing Translational Sciences/National Institutes of Health)*

Pei-Hsuan Chu described a highly reproducible, automated, high-throughput gene expression detection platform used by researchers at the National Center for Advancing Translational Sciences (NCATS). This platform, known as *RNA-mediated oligonucleotide Annealing, Selection, and Ligation with Next-Gen sequencing* (RASL-seq) bypasses the cDNA synthesis step that contributes the most variation between assays, to obtain a direct measurement of relative abundance of target transcripts.

With the advent of next-generation sequencing (NGS) technologies, genome-wide RNA sequencing (RNA-seq) and targeted sequencing methods have recently been exploited for comprehensive transcriptome analysis. However, despite the genome-

wide information provided by RNA-seq methods, some issues remain with the current gene expression assays. These include the biases introduced during library preparation, as well as inter-experiment variability. The cost of RNA-seq remains very high, and therefore limits the number of doses, replicates and compounds analysed.

The RASL-seq method is a new approach that can be applied to toxicogenomics, a field of science that encompasses toxicology, genetics, molecular biology and bioinformatics, to describe the response of organisms to chemical exposure. Toxicogenomics not only reveals mechanistic information of observed toxicity, but also captures the early stages of adverse events that may not show in the endpoints of cell-based assays.

The RASL-seq method used at NCATS is based on a model originally developed by Li *et al.* (49). This technology, which takes advantage of NGS, bypasses the isolation of RNA from samples and the cDNA synthesis step, permitting quantitative profiling of several hundred selected genes in a large number of samples. To enable full automation and avoid the uneven loss of magnetic beads during liquid handling, the assay switches to solid state (oligo-dT coated plate) rather than magnetic beads. The DNA probe annealing step was separated from the cell debris to minimise unspecific interactions due to the lysis buffer, genomic DNA or other cell debris. The assay provides direct analysis of RNA levels in cell lysates and is adaptable to full automation. RASL-seq has proven highly reproducible, as well as low in cost.

NCATS researchers are employing an improved RASL-seq platform, illustrated in Figure 16, to gather dose–response and time–response data to help show the toxic mechanisms of various compounds. This improved method, the Turbo RASL-seq procedure, currently multiplexes for 347 genes, up to three probes per gene per sample, and gathers information for 384 samples in one sequencing reaction. The very high reproducibility of this assay enables the study of compound effects over multiple time points with full dosage coverage. NCATS has also developed a quantitative analysis method to elucidate the dose–response relationships for each gene upon treatment and identify the Bench Mark Dose (BMD) and Point of Departure (POD).

NCATS is currently applying the RASL-seq assay to screen tobacco toxicants, by using immortalised cells and induced pluripotent stem cell (iPSC)-derived endothelial cells, to determine how smoke-derived chemicals interact with human cells by their genetic alteration. In a recent study, the expression of 347 stress genes was measured in human umbilical vein endothelial cells (HUVECs) exposed to 18 tobacco components (50). Detailed dose–response relationships were established for each gene by using the quantitative analysis method to identify the BMD and POD.

The RASL-seq method produced highly reproducible gene expression data with high-throughput and low cost to quantitatively assess the adverse effects of the individual chemical components of tobacco smoke. The computational method identified the BMD and POD based on the change of gene expression over toxicant concentration for each gene and treatment. These measures quantified the dose-dependence of gene responses, and enabled a pathway analysis to clarify the mode of action of each toxicant. To summarise, the key points are:

— The RASL-seq gene expression platform achieves high-throughput, high quality gene expression data at low cost.

— Quantitative gene expression data enables the identification of BMD and POD for risk assessment.

— POD heat maps can condense gene expression information from multiple dosages and time points, and reveal both the potency and trend of expression changes.

Predicting Exposure Response in the Airway: Integrating Cellular Signalling and Epigenetics Through the Epigenetic Seed and Soil Model of Interindividual Variability *(Shaun McCullough, US Environmental Protection Agency)*

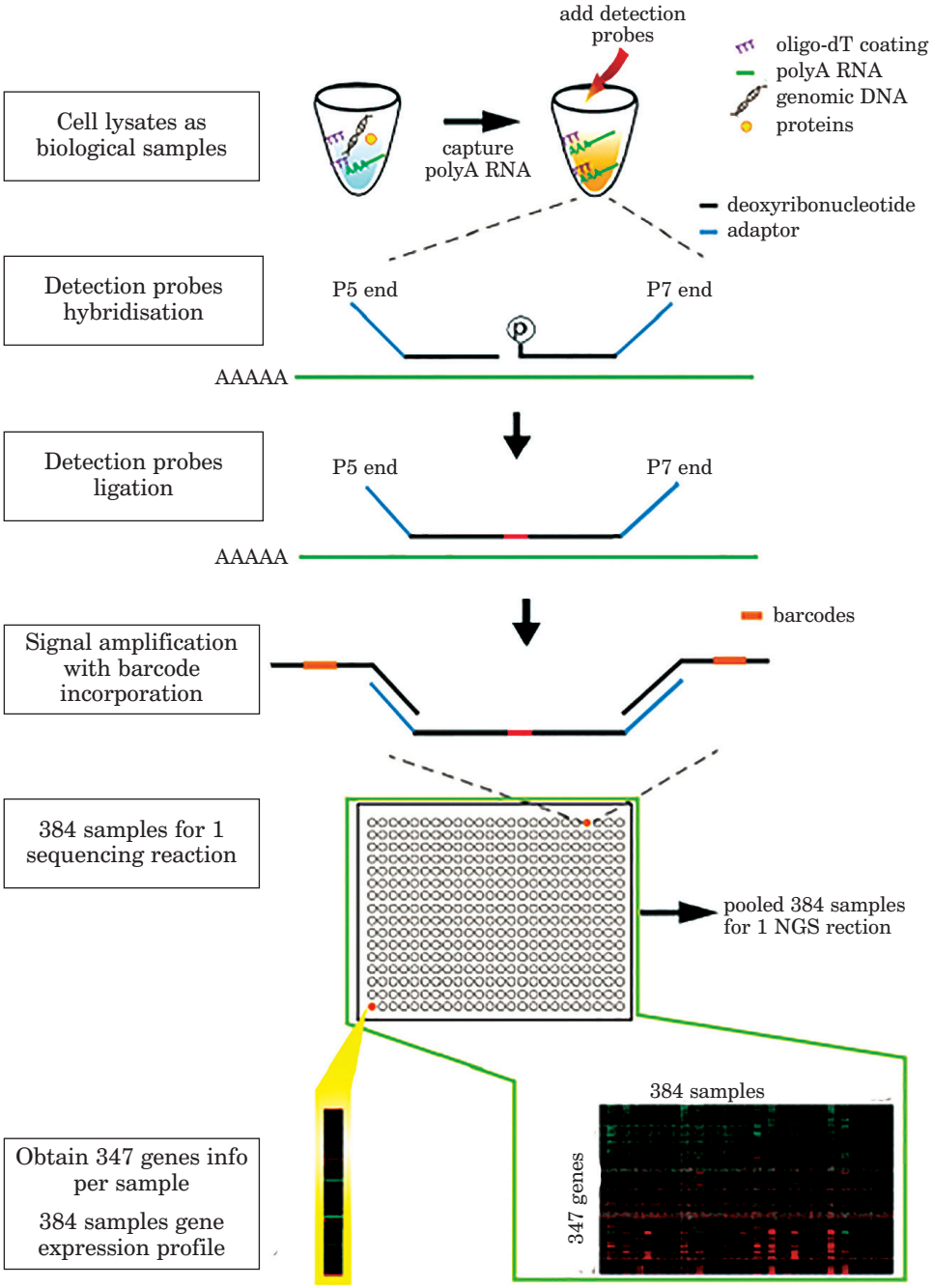
Shaun McCullough explored the field of epigenetics and its potential for transforming our understanding of inter-individual variability in response to toxic exposures.

Despite the advancement of many approaches that examine the role of histone modifications, a key aspect of the epigenome, relatively little has been done to date to examine the role of the epigenome in exposure effects and susceptibility *in vitro*. The broad range of responsiveness between individuals to air pollutant exposure has not been well explained by current susceptibility models, and the mechanisms underlying this inter-individual variability remain elusive. Traditional susceptibility markers do not faithfully explain variability and gene variants do not completely explain susceptibility.

The epigenome and its role in inter-individual variability is becoming an important consideration in toxicology and risk assessment. The epigenome is essentially a 'master regulator' of gene transcription. It is characterised by heritable factors that regulate gene expression without changing the DNA sequence. That is, the epigenome alters how genes are used without altering the genes themselves — changing phenotype without changing genotype.

The framework of the epigenome is chromatin, which is composed of nucleosomes, the basic units of DNA packaging. Each nucleosome has eight histone proteins (two each of histones H2A, H2B, H3 and H4), joined together by linker histones. Histone 'tails' extend out of each histone and can serve as substrates for modifications by acetylation, methylation and other chemical groups. Epigenetic regulators, such as chromatin modifications and DNA methylation, function as critical and dynamic mediators of gene expression and shape how cells, tissues and individuals respond to their environment. In toxicology, the role of the epigenome has mainly been explored by looking at changes in DNA methylation in response to exposures. However, more recent findings indicate that histone modification patterns can predict variability in gene expression, and have implicated histone

Figure 16: The Turbo RASL-seq procedure



Method modified from Li, H. et al. *(49).*

modifications as susceptibility factors in a number of diseases.

Researchers at the Environmental Protection Agency carried out a study to test whether inter-individual variability in basal and toxicant-induced gene expression resulted from differences in baseline patterns of chromatin modifications that existed prior to exposure (51). Specifically, they sought to determine whether baseline levels of certain chromatin modifications correlated with the inter-individual variability in ozone-mediated responses in an air–liquid interface model of primary human bronchial epithelial cells. Ozone is a model air pollutant that induces the expression of pro-inflammatory mediators and markers of oxidative stress both *in vitro* and *in vivo*. The airway epithelium, the barrier between the lung and the environment, plays a critical role as a modulator of pro-inflammatory and oxidative stress in response to environmental exposures.

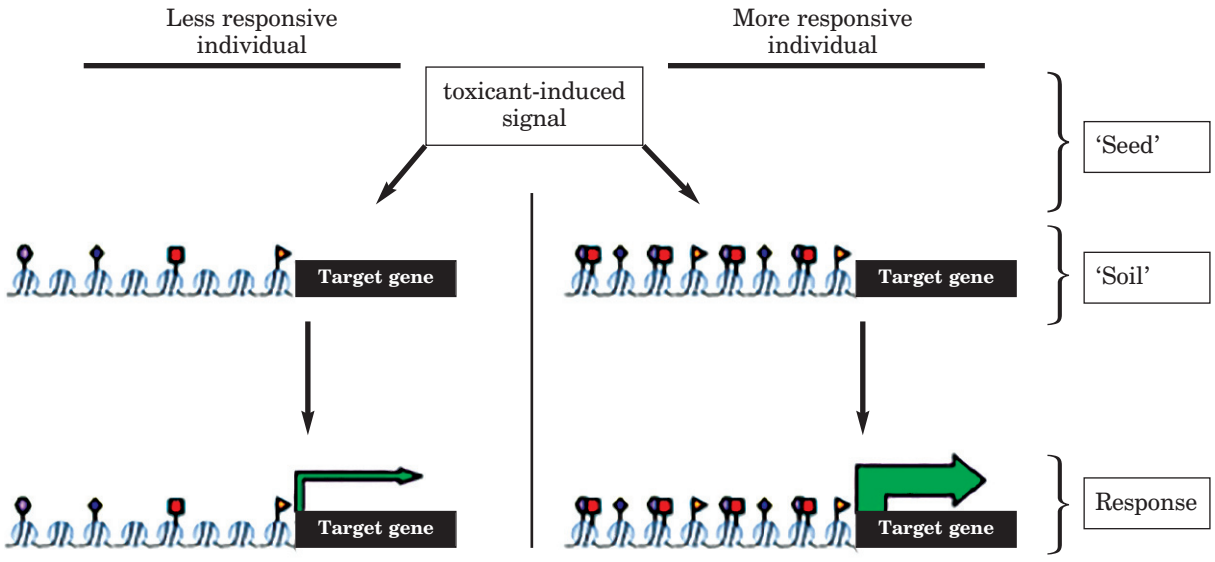
During the course of this study, two distinct mitogen activated protein kinase (MAPK) pathways (EGFR/MEK/ERK and MKK4/p38) were identified as the drivers of the cellular response to ozone (52). This varies from traditionally accepted findings in cell lines implicating the NF-κB pathway. While the ozone-mediated induction of pro-inflammatory cytokines (IL-8, IL-6, COX2, IL-1α, and IL-1β) varied between donors, the relative activation of MAPK signalling was similar. Given this similarity, the researchers hypothesised that the variability in responsiveness originated downstream of MAPK signalling, specifically in the patterns of chromatin modification within the regulatory regions of target pro-inflammatory genes.

The results showed that pre-exposure patterns of chromatin modifications (histone H3 lysine 4 trimethylation, H3 lysine 27 di/trimethylation and 5-hydroxymethylcytosine) correlated with the magnitude of post-exposure pro-inflammatory gene expression. These findings highlighted the utility of advanced *in vitro* models in modern mechanistic and epigenetic toxicology. They also contributed to the establishment of physiologically relevant *in vitro* models as the foundation of the emerging field of epigenetic toxicology.

In summary, the researchers proposed an 'epigenetic seed and soil' model to describe the epigenetic basis of inter-individual variability in exposure responses, as shown in Figure 17. In this model, toxicant-induced cellular signals (the 'seed') interact with the chromatin landscape in the nucleus to alter the expression of toxicant-responsive genes. Specific chromatin modifications (the 'soil') prior to exposure vary between individuals. These intrinsic variations in the soil regulate the magnitude of exposure-related gene induction. The key points are:

— Epigenetic regulators function as critical and dynamic mediators of gene expression and shape the way cells, tissues and individuals respond to their environment.

Figure 17: The seed and soil model for the epigenetic basis of inter-individual variability in exposure responses



— The emerging field of epigenetic toxicology will ultimately play a critical role in our understanding of exposure-associated health effects and susceptibility.

— The epigenome has the potential for being a transformative tool for risk assessment.

Breakout Discussion Groups

Overview

Moderated breakout groups were held in two subject areas: *In Vitro Exposure Systems* and *Dosimetry*. All groups had the same goals:

— To understand the advantages and weaknesses of each of the major *in vitro* exposure systems.

— To define the major points to consider when extrapolating from *in vitro* to direct human exposure.

— To understand the advantages and drawbacks of the various dosimetry tools.

— To identify the limitations of the current *in vitro* exposure systems and dosimetry tools and propose activities to address these limitations or gaps.

The *In Vitro* Exposure Systems Group was provided with the following set of questions to guide the discussions:

a. What is the utility domain of each exposure system?

1. Is it useful for both particulate and gaseous exposure?
2. Is it limited to certain physical configurations of cells or tissues?
3. Is it adaptable to different smoking regimens?
4. Can different model types be exposed?
5. What are the major shortcomings of the system?

b. How well-characterised is the exposure system?

1. Ease of use and throughput?
2. Available to other laboratories?
3. Portability to other laboratories?
4. Interlaboratory reproducibility established?
5. Used with tobacco-related materials (chemicals)?

c. What research activities can be proposed to address any gaps and limitations?

The Dosimetry Group was provided with the following questions to guide the discussions:

a. What is the state-of-the-art for in vitro dosimetry measurements?

1. What measurements allow the most useful comparisons to be made with historical toxicology studies?
2. What measurements allow the most useful comparisons to human clinical studies?
3. What measurements are sufficient for e-cigarettes?
4. What measurements are sufficient for heat-not-burn tobacco products?
5. Are there measurements that should be performed to assess the exposure to all types of tobacco products?
6. Should thresholds be placed on limits of detection?

In Vitro Exposure Systems

Assumptions

In discussing exposures, the breakout group made the following assumptions:

Products to address include:

— tobacco-related products (cigarettes, tobacco heated products, cigars, cigarillos);

— nicotine delivery devices (e-cigarettes);

— other products (e-shisha, water pipes);

— possibly other aerosols/particles (nanoparticles).

The methodology would be:

— of value to regulatory community and research;

— *in vitro*;

— compatible with good laboratory practices (transferable between laboratories; characterisation, robustness, repeatable, flexible).

Aerosol generation

The following should be addressed when considering the generation of aerosols:

— regulatory testing and basic research applications;

— reproducibility;

— available dosimetry tools;

— transferability between laboratories;

— flexibility to generate smoking regimes and/or other human smoking profiles.

Dilution principle

The following should be addressed regarding dilution principles:

— available dosimetry tools;
— transferability between laboratories;
— flexibility for aerosols;
— good mixing, minimal dead space;
— dilution working range (to be defined);
— quantifiable retention time;
— controlled air source.

Cellular exposure modules

The following should be addressed in preparing cellular exposure modules:

— focus on tobacco and nicotine delivery products;
— direct exposure to biological test systems;
— available dosimetry tools;
— must be able to determine dose at tissue surface;
— transferability between laboratories;
— uniform exposure within module;
— flexibility for aerosols;
— minimal effects of 'exposure physics' on cells;
— easily cleanable/ reusable;
— flexibility for different cell/tissue types/insert.

Study design

The following should be addressed in designing an exposure study:

— ALI exposures should include 'sham', incubator, and positive and negative controls;
— good cell culture must be practised;
— replicates should be reported;
— dynamic exchanges should be implemented if media is exposed to aerosol;
— insert quality/type must be considered;
— endpoints should reflect the pending question being addressed.

Dosimetry

The breakout group focused on addressing Item #22 of the 56 research priorities established by the Center for Tobacco Products, FDA, in January 2012: "What *in vitro* and *in vivo* assays are capable of comparative toxicity between two different tobacco products; with special attention to cardiotoxicity, respiratory toxicity, carcinogenicity, and developmental/reproductive toxicity?" The aim was that any findings and recommendations should support comparisons of exposure, dosimetry and biological end effect between new and predicate products.

Areas in the in vitro *exposure system where dosimetry is most important*

— Dosimetry techniques should provide an understanding of what is dosed onto the test system, including its representative chemical profile and its quantity.
— At the site of test system exposure, current systems include surrogate targets like QCM, cell-containing or cell-free inserts, and media.
— Dosimetry techniques support a reasonable comparison of new and predicate product exposures. Applying dosimetry immediately after puff release from the tobacco product allows further comparison of chemical profiles between new and predicate products.
— Utilising the standardised historical approaches for chemical/particulate capture (Cambridge filter pads, impinged aqueous and organic fractions, etc.) supports vital historical data bridging.
— Characterising the smoke/vapour generator and exposure system is critical. Elucidation of the impacts of the system on the vapour/ smoke chemistry may also help in optimising: a) future exposure platforms to minimise artefacts; and b) exposures that better mimic the exposures at specific sites along the human respiratory tract.

Substances and factors that can be analysed in various product types

Identifying and standardising analytes is an important future goal. Some product types discussed by the breakout group include the following:

— non-nicotine devices (glycerol);
— e-cigarettes (nicotine, glycerol), but there is an absence of a standard puff profile (a CORESTA profile is available for one unique device);
— heat-not-burn (HNB) products (nicotine, particle size);
— combustible products, with reference cigarettes as a basis, to determine TNCO (tar, water nicotine and carbon monoxide), TSNAs (tobacco-specific nitrosamines), reactive aldehydes, and puff profiles, standardised by ISO/HCI.

This list is not fully exhaustive and needs further development.

Questions to consider when comparing products

New *versus* reference/predicate products:

— Was the dosimetry sufficient to support the conclusions of the assay or study?

— What gaps exist in '*in situ*' measurement (i.e. how do we ensure target tissue dosimetry?)

Future considerations

— Improve the ability to measure gas/vapours immediately at the ALI, without interfering with exposure.

— Use specific 'sensors', such as ROS sensors. Consider including non-cellular chemical reactions for detection.

— Explore target cell-specific dosimetry, i.e. cell–molecule interactions. Determine whether this is needed for regulatory submissions.

— Be amenable to various platforms including high-throughput systems.

— Ensure compatibility with new test system technologies (i.e. lung-on-a-chip).

— Use models of *in vivo* exposures to optimise the *in vitro* exposure systems and associated dosimetric tools.

Discussion

The overarching goal of the workshop was to establish a platform for internationally recognised experts to convey the current state of *in vitro* smoke and aerosol/vapour exposure systems, the various approaches and challenges to quantifying the complex exposures, and for participants to propose solutions to advance these technologies for the evaluation of new tobacco products.

The workshop format was designed to progress from presentations on background information, through detailed discussions to final recommendations for ways forward. Introductory speakers first relayed efforts at the FDA to advance regulatory toxicology (with a focus on non-animal approaches), followed by a review of issues with *in vitro* to *in vivo* extrapolation, inherent variability across animal models, and the challenges for *in vivo*/*in vitro* correlations. For the audience, this background information helped establish why animal models have not provided adequate data to fully understand how humans are impacted by inhalation exposures. The four core subjects (Tobacco Smoke and E-Cigarette Aerosols; Air-Liquid Interface-*In Vitro* Exposure Systems; Dosimetry Approaches for Particles and Vapours/*In Vitro* Dosimetry Determinations; Exposure Microenvironment/Physiology of Cells) were arranged in a logical manner that took the listener from the chemistries of cigarettes and tobacco-based inhaled products, to modern exposure systems used to deliver them, to dosimetry approaches used to quantify them, and finally, to an overview of the types of *in vitro* tissues exposed and how they respond. A final segment on promising technologies provided the audience with examples of what can be expected of *in vitro* systems in the near future.

The poster presentations and ample networking opportunities fostered candid participant interactions, allowing new opportunities for stakeholder research and collaboration to be explored. Informal discussions, as well as speaker panel Q&A sessions, focused on existing technologies, approaches to solving problems, and reiteration that collaborative efforts would be the best mechanism for advancing the science. The breakout group conclusions identified priority areas that should be addressed for both exposure systems and dosimetry.

A broad and freely participatory audience demonstrated the general enthusiasm for the topics covered in this second-in-series IIVS workshop. Statements by the workshop attendees affirmed their substantial interest, and the agreed necessity of using *in vitro* methods to assess adverse human health effects by inhalation-based MRTP. Building on relevant topics previously identified by the various stakeholders, the workshop participants addressed research areas needing attention for *in vitro* systems to offer solutions for the FDA/CTP priorities (53).

Next steps

Prevalent throughout the breakout group sessions and informal workshop discussions was the widespread acknowledgement that standardised methods need to be used in exposure paradigms that most closely reflect consumer use patterns. This need for standardised methods also extends to dosimetry approaches, given the detection variabilities reported by different laboratories. However, as with the first IIVS workshop, it was recognised by the attendees that progress will be slow in the absence of relevant funding mechanisms to assist the development of *in vitro* systems useful to industry, independent research laboratories and regulatory scientists. However, these systems will be necessary since they will provide meaningful and human-relevant data to support decision-making processes. Additionally, collaborative, inter-laboratory efforts that incorporate a transparent process (ideally involving regulatory scientists) will be needed to accelerate the acceptance of such technologies. This will be accomplished by identifying studies to be run by specific laboratories employing exposure

systems or analytical equipment designed to quantify the materials of interest. Although exposure systems may have varied designs, the underlying need is to understand and quantitate exactly what commonly used smoke and aerosol generators create. It is essential to know the composition of the smoke, or aerosol, that is applied to *in vitro*/*ex vivo* tissues. Only once the accurate dose of potential harmful materials has been quantified, can effective dose–response relationships be established for *in vitro* pulmonary models. This will then allow more accurate extrapolation between *in vitro* and *in vivo* data sets; arguably an essential component for *in vitro* data to support potential regulatory decision processes.

Summary

The *In Vitro Exposure Systems and Dosimetry Assessment Tools for Inhaled Tobacco Products* workshop described here was considered a success in bringing together experts and stakeholders in a vibrant programme that addressed topics in alignment with the FDA-CTP's mission. It yielded a path forward that identified key elements that need to be incorporated into the use of exposure systems and the dosimetry techniques used to quantify materials generated by them. Addressing these key elements will involve engineering principles that will need to be aligned with dosimetry requirements to accurately create exposure scenarios intended to model representative human usage and resulting lung effects from inhaled products. These activities all support the identification, validation, and dissemination of robust *in vitro* methods for the evaluation of tobacco products and their constituents, a process necessary for modernising and advancing regulatory decision-making to protect human health.

Summary of key themes

1. Creating accurate and dependable means to quantify pulmonary exposures to inhaled materials, including tobacco-related mixtures and constituents, is challenging. Developing and validating new test methods for use in a regulatory safety testing arena requires the input and guidance from multiple stakeholders.

2. E-aerosols are physically similar to cigarette smoke in some aspects (e.g. size range), but chemically very different. Systems designed for cigarette evaluation may not be compatible with the *in vitro* testing of e-aerosols. Smoking machine requirements for conventional and electronic cigarettes are different, but share the same basic technologies.

3. Tobacco products, such as cigarette smoke and e-cigarette aerosols, are capable of generating reactive oxidants.

4. There are a variety of strategies for exposing cells at the ALI, starting from acute toxicity studies (dose–response relationships), up to repeated exposure studies at non-toxic doses. The choice will depend on the protocol, and a clear definition of what is to be analysed and demonstrated.

5. Mathematical dosimetry modelling, simulations with CFD-based models, and microfluidic airway models based on primary human cells offer realistic approaches for studying the fate of inhaled chemicals and the links between exposure characteristics and biological responses.

6. The emerging field of epigenetic toxicology will ultimately play a critical role in our understanding of exposure-associated health effects and susceptibility.

7. Accurate dosimetry data can support the results from *in vitro* toxicity of cigarette smoke.

8. The next generation products category will continue to grow, evolve and diversify and dosimetry will support exposure.

Acknowledgements and Disclaimer

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Appendix

Table A1: Abbreviations list

ACM	aerosol collected mass
ALI	air–liquid interface
AOP	adverse outcome pathway
AUC	area under the curve
BAT	British American Tobacco
BMD	benchmark dose
CFD	computational fluid dynamic
CMAG	condensation monodisperse aerosol generator
COPD	chronic obstructive pulmonary disease
CTP	Center for Tobacco Products (FDA)
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GC × GC	two-dimensional gas chromatography
GVP	gas vapour phase
HCI	Health Canada Intense
IIVS	Institute for In Vitro Sciences
ISO	International Organisation for Standardisation
ISDD	*in vitro* sedimentation diffusion dosimetry
LADD	lifetime average daily dose
MPPD	multiple path particle density
MTD	maximum tolerated dose
NCATS	National Center for Advancing Translational Sciences
NCCT	National Center for Computational Toxicology
NHLBI	National Heart Lung and Blood Institute
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NOAEL	no observed adverse effect level
NRC	National Research Council
NRU	neutral red uptake
OX/ROS	oxidants/reactive oxygen species
PCLS	precision-cut lung slices
PBPK	physiologically based pharmacokinetic
POD	point of departure
QCM	quartz crystal microbalance
RASL-seq	RNA-mediated oligonucleotide annealing, selection and ligation with Next-Gen sequencing
RHuA	reconstructed human airways
ROS	reactive oxygen species
SEIVS	smoke exposure *in vitro* system
TOF-MS	Time-of-flight mass spectrometry
Tox21	Toxicology in the 21st Century
TPM	total particulate matter
ZTRI	Zhengzhou Tobacco Research Institute